AD_____

Award Number: DAMD17-99-1-9250

TITLE: Expression Profiling of Tyrosine Kinase Genes

PRINCIPAL INVESTIGATOR: Heinz-Ulrich Weier, Ph.D.

CONTRACTING ORGANIZATION: University of California at Berkeley
E. O. Lawrence Berkeley National Laboratory
Berkeley, California 94720

REPORT DATE: August 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

20030203 048

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE August 2002	3. REPORT TYPE AND DATES COVERED Annual (1 Aug 01 -31 Jul 02)

4. TITLE AND SUBTITLE
Expression Profiling of Tyrosine Kinase Genes

5. FUNDING NUMBERS
DAMD17-99-1-9250

6. AUTHOR(S):
Heinz-Ulrich Weier, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California at Berkeley
E. O. Lawrence Berkeley National Laboratory
Berkeley, California 94720

E-MAIL: UGWeier@lbl.gov

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The expression of genes involved in signal transduction (e.g. protein kinases) is often altered in tumors. The aberrant expression of several of these genes typically parallels the progression toward a more malignant phenotype. We developed a cDNA micro-array – based screening system to measure the level of expression of tyrosine kinase (tk) genes. The hardware for preparation of cDNA micro-arrays and basic protocols for hybridization were developed in year 1. In the second year, we finished isolating RNA and cDNA synthesis from 16 breast cancer cell lines and 10 frozen tissues. We optimized protocols for tk-specific PCR amplification and cloning. We continued our DNA sequencing effort and added additional targets to our micro-arrays. Using well-characterized breast cancer cell lines, the system delivered reproducible results about tk gene expression during cell transformation and progression toward a more malignant phenotype. Comparing the absolute expression levels from cDNA micro-arrays with data from Northern blot analyses suggested that our initial approach using mixed-based oligonucleotide primers led to lowered representation of highly abundant transcripts. This problem has been addressed with a new oligonucleotide primer design and a modified DNA amplification protocol that we now apply to investigate the tk gene expression in small tissue samples.

14. SUBJECT TERMS breast cancer, gene expression, tyrosine kinase	15. NUMBER OF PAGES 60
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

INTRODUCTION:

Aberrant expression of receptor or cytosolic tyrosine kinase genes and, in particular, their hyper-expression are common phenomena in breast cancer, which are believed to alter cell growth and response to external signals such as growth factors, hormones etc. Knowledge about the relative levels of expression of many tyrosine kinase genes, all at the same time, might contribute significantly to a better understanding of the processes of tumor development and progression. We are developing a rapid assay that will use innovative cDNA micro-arrays carrying small amounts of individual tyrosine kinase gene-specific targets to simultaneously determine the expression levels of up to 100 tyrosine kinase genes using a small number of cells. Our research and development will lead to the definition of a set of gene-specific markers associated with breast cancer progression and a simple device capable of performing inexpensive expression profiling of these markers. The research and development efforts in the first 2 years of this project focused on the design and testing of robotic instruments to prepare DNA micro-arrays and the preparation of prototype arrays carrying sets of more than 50 gene-specific tyrosine kinase fragments. The third year effort (i.e., the reporting period 1 Aug 01 –31 Jul 02) was directed towards the optimization of in vitro DNA amplification protocols and validation of results from cDNA micro-array experiments.

During this third reporting period, Dr. Weier's effort has averaged 15%. This reduction in effort was due to Dr. Weier being on leave under the federal Family Medical Leave Act of 1993 (FMLA) since November 2001 due to his wife's disability and the birth of their daughters. A postdoctoral fellow, Lisa Chu, Ph.D., was hired in January 2002 to assume some of the duties. In February of 2002, LBNL notified the Commander, U.S. Army Medical Research and Material Command, that the Laboratory is making a one-time extension through June 30, 2003 in accordance with Article 5, Approvals and Other Authorizations. This no-cost extension was approved by the Grants Officer, U.S. Army Medical Research Acquisition Activity on March 19, 2002 to complete the research as proposed.

BODY:

Here, we report our progress as it relates to the approved 'Statement of Work'.

Task 1. **Identify tyrosine kinase (tk) genes expressed in normal and neoplastic breast tissues (Months 1-24)**

and

Task 2. **Measure tk gene expression in cell lines, normal and tumor tissues (Months 3-24)**

Completed as proposed and described in previous two progress reports.

Briefly, we isolated total RNA from 16 different breast cell lines provided by our collaborators Paul Yaswen, Ph.D., Martha Stampfer, Ph.D., Daniel Callahan, Ph.D., and Ruth Lupo, Ph.D., from LBNL, and Chris Benz, M.D., from the Buck Institute for Aging Research, Novato, CA. (Table I). Normal breast tissue and breast cancer tissue specimens were obtained from the University of

California Comprehensive Cancer Center (Joe W. Gray, Ph.D., B.M. Ljung, M.D., and K. Chew). We received a total of 10 frozen tissue specimens representing 3 normal tissues and 7 cancer tissues.

Table I. Breast cancer cell lines used in our experiments.

ZR75	SKBR3
BT474	BT468
BT549	MCF-7
184A1	184A1TH-6
184B5	184B5-ME
T47D	MDA-MB-453
MDA-MB-436 plus Vit. A	MDA-MB-436 plus pCMr3.1
600MPE	HMEC

RNA was extracted using a commercial kit (Qiagen) and transcribed into cDNA immediately after isolation. Remaining RNA was stored at –80 degrees. We prepared cDNAs from the RNA by random priming and reverse transcription. Commercial kits (Qiagen, Roche, Ambion) were used for all steps. Typically, 1 μg of total RNA produced sufficient quantities of cDNA for cloning and/or repeated micro-array analyses.

CDNA micro-arrays were prepared in-house on poly-L-lysine coated slides and hybridized with CY3- and CY5-labeled probes. CDNA probes prepared from other tissues or cell lines such as thyroid cells lines known to express a particular tk gene at high levels were used as controls. The second year effort (i.e., the previous reporting period) was directed towards the optimization of cDNA preparation, labeling, hybridization and detection protocols as well as the molecular cloning and sequencing of breast cancer-specific tyrosine kinase gene transcripts.

The DNA from plasmid clones with inserts of about 125-190 bp was isolated, fingerprinted or screened against known tk genes and 'novel' clones were sequenced at the UC Berkeley, Biochemical Core Facility. The list of genes cloned and identified contained several sequences reported to have transforming activity, such as brk, trk, axl/ufo or to be overexpressed in various types of cancer. Our present panel of kinase gene tags used to prepare cDNA micro-arrays contains 60 genes among them four novel sequences and HLA-A, which happens to be amplified by our PCR primers.

To search for full length cDNA clones for some of the novel sequences found expressed in breast cancer, we employed the services of a local company (Pangene of Fremont, CA). Pangene used two of our sequences to screen their proprietary cDNA libraries for full length clones. In first attempts, the expressed sequence tags (EST's) were too short to isolate a cDNA clone. However, one EST could be mapped to chromosome 1 by database searches, and expanded by using flanking genomic sequences. This provided sufficient sequence information for screening and we obtained one cDNA clone with a 5.5kb insert sequences in the 6.5 kb of vector plasmid pEAK8. Exactly 1,316bp of the clone were analyzed by sequencing. 1,248 bp of 1,316 bp were identical (~100%) to the exons 72-74 of heparan sulfate proteoglycan 2 (HSPG2).

Heparan sulfate proteoglycan 2 (HSPG2) is the major heparan sulfate proteoglycan of basement membranes and the gene located on chromosome 1p36.1. It possesses angiogenic and growth-promoting attributes primarily by acting as a coreceptor for basic fibroblast growth factor (bFGF). BFGFs have diverse functions in cell growth and differentiation. In addition, multiple roles have been proposed for bFGFs in cancer origin and progression. BFGFs signal through transmembrane receptor tyrosine kinases. This signalling is regulated by a balance of HSPGs that either stimulate or

inhibit bFGF binding to its bFGF receptor. Even though HSPG2 is not a tyrosine kinase, the involvement of HSPG in breast cancer progression as reported in the literature makes this gene a worthwhile addition to our expression panel. However, Pangene Corp. was not able to provide a cDNA clone for our second sequence of interest. We have therefore initiated a collaborative effort with Dr. Melvin Simon at the California Institute of Technology, Pasadena, CA, to screen the BAC libraries for genomic clones containing our genes of interest.

Task 3. **To <u>validate</u> assays for multigene expression profiling in small amounts of tissue (months 18-36)**

In accordance with the approved milestones our efforts in this third reporting period focused on improving system parameters to allow tk gene fragment amplification from small tissue samples and validation of micro-array results.

Task 3a. Develop software for databasing, automated analysis of expression profile data sets and their annotation (months 18-24)

Given the rather small number of cDNA samples analyzed to date, we decided to handle all results in form of Microsoft Excel spreadsheets. We will revisit the need for automated analysis of hybridization results in the coming year, when we plan to analyze a much larger number of tissue samples obtained by tissue microdissection.

Task 3b. Test the system with serial dilutions of cells (months 24-30)

The RT-PCR approach using highly degenerate (mixed base) primers may be the method of choice to identify novel tk genes expressed in these tumor, but it led to non-linear amplification of individual cDNA species. Expression levels measured with the cDNA micro-array method differed from those estimated by Southern blot analyses, and generally showed a concordant trend (i.e., up- or down-regulated expression), but a reduced dynamic range. Furthermore, serial dilution of RNA suggested that the reverse transcription polymerase chain reaction (RT-PCR)-based scheme led to reproducible results, if the equivalent of 500-1000 cells was used as amplification template. We attributed this to the fact that the original set of mixed-base oligonucleotide primers were designed based on the conserved amino acid sequence and were comprised of 1024 and 6144 different sequences for the forward and reverse primer, respectively. This high complexity of PCR primers had two consequences: 1. the concentration of any one particular primer sequence was very low, and 2. forward and reverse primers were used in different concentrations. We therefore redesigned our PCR primers using the published cDNA sequences of known tk genes. We then optimized the PCR amplification scheme carefully balancing the amounts of forward and reverse primers and we optimized primer annealing conditions. Although the new primer set is similar to the previous set, the complexity of the new primers was decreased 1.8-fold for the new forward primer and 3-fold for the reverse primer. This is expected to lead to increased specificity and amplification efficiency. Next, we developed a 2-step amplification protocol in which the mixed base primers are used only

during the first 5 cycles, when the PCR product are generated mostly from the cDNA templates. The in vitro DNA amplification is then continued with a second set of non-degenerate oligonucleotides, which anneal to the 5'-ends of the PCR products. Initial measurements to test the linearity of the amplification process using artificial mixtures of cloned tk gene fragments have shown superior (i.e., more linear) amplification results.

Task 3c. Test the assay with micro-dissected tissue from breast cancer sections

This task required improvements in our in vitro DNA amplification protocol and will be addressed in the coming year, i.e., in months 37-48.

Validation of cDNA micro-array data

We applied fluorescence in situ hybridization (FISH) with non-isotopically labeled probes to validate micro-array results. Initially, we used dual color FISH with cDNA probes on paraformaldehyde-fixed breast and thyroid cancer cells. A typical example, the demonstration of ret expression in MCF-7 (breast cancer) and TPC-1 (thyroid cancer) cells is shown in Figure 1. Figure 1 also shows the high levels of expression of brk in MCF-7 cells, but low level expression in TPC-1 cells.

To be able to analyze the expression level of 5 genes in situ, we developed a FISH protocol with Spectral Imaging analysis. Funding provided by an NIH grant allowed the purchase an Applied Imaging, Inc., (ASI) Cytogenetics Workstation. The system comprised of a Sutter, Inc., xenon light source, Sutter, Inc.'s 'Liquid Light Guide', an ASI Interferometer, and MS Windows compatible computer was installed in the P.I.'s lab. in February of 2002. The system allows us to record entire fluorescence spectra with a resolution of about 10nm for each pixel in the image. With a software package provided by ASI (SpectraView), we were able to deconvolute the spectra and calculate the relative amounts of bound probe. In preliminary experiments, we tested this approach for cDNA micro-array validation on breast cancer cell lines and mixtures of breast cancer cell and thyroid cells. Figure 2 shows the typical analysis. A mixture of MCF-7 cells and TPC-1 cells was hybridized with a combination of 5 different cDNA probes. Each of the cDNA probes was labeled with a unique reporter molecule. Following hybridization and removal of unbound probes, cellular DNA was counterstained with DAPI. We then recorded DAPI images using a DAPI-specific excitation and emission filter set, and an entire fluorescence spectrum (400nm – 800nm) with a multiband excitation and emission filter set. The SpectraView allowed us to deconvolute the image and extract 5 images representing the contribution of each of the 5 dyes used. We defined regions of interest and calculated the relative intensity of each cDNA probe. In these preliminary studies, we were able to confirm expression levels suggested by our cDNA micro-array studies.

In the next year, we plan to optimize cell fixation and hybridization protocols, and apply the technique for cell-by-cell tk gene expression studies on tissue section cut from breast cancer specimens.
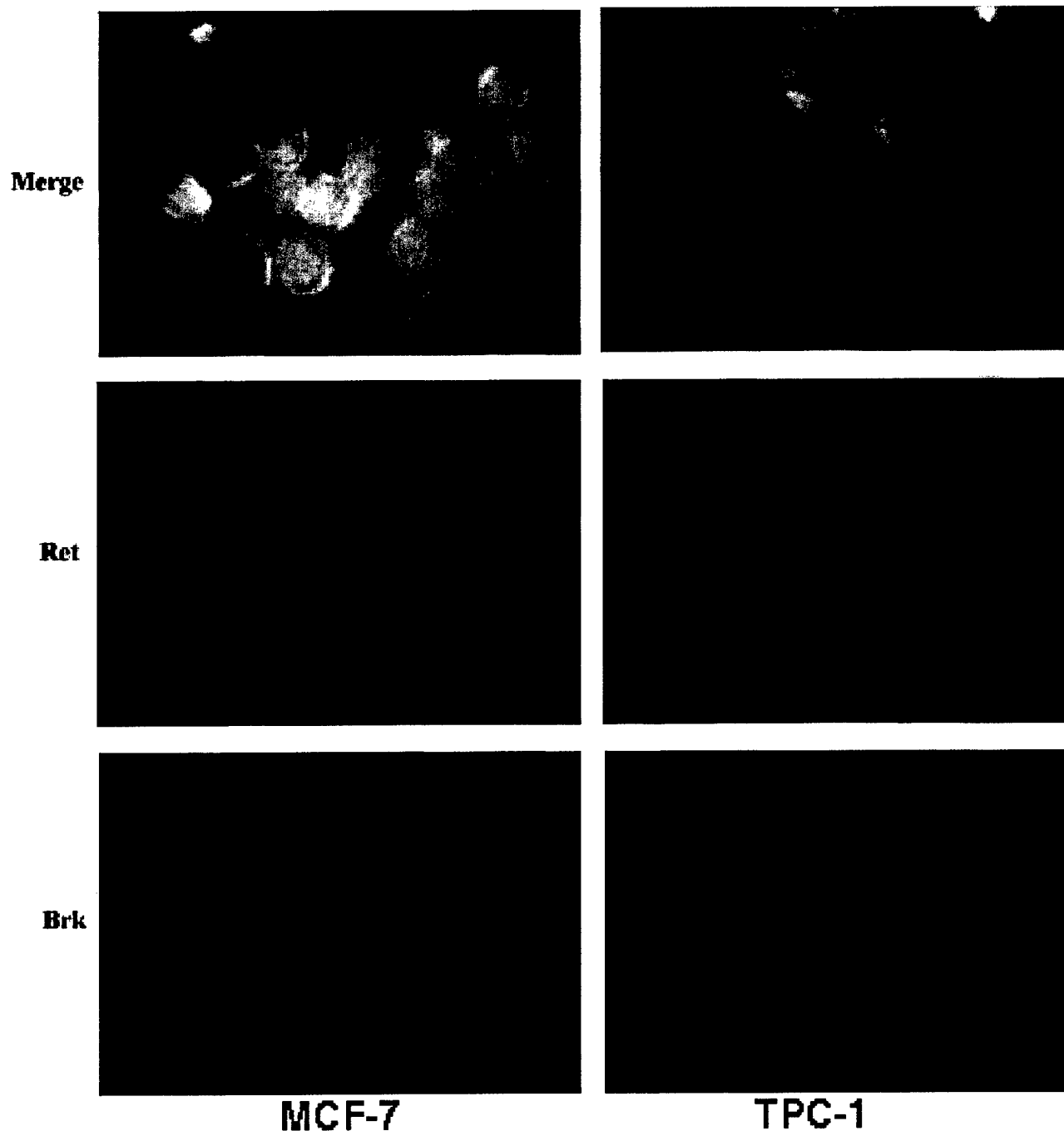
Figure 1: Visualization of gene expression by non-isotopic in situ hybridization. Cells from the breast cancer cell line MCF7 (panels to the left) and the thyroid cancer cell line TPC-1 (panels to the right) were hybridized with a combination of a green fluorescent cDNA probe for the oncogenes ret and a red fluorescent probe for brk. Both cell lines showed high levels of expression of ret (middle panel), but only MCF-7 cells showed elevated levels of brk expression (lower left). The top panels show merged images of DAPI counterstained cells (blue fluorescence) and the red/green hybridization results.
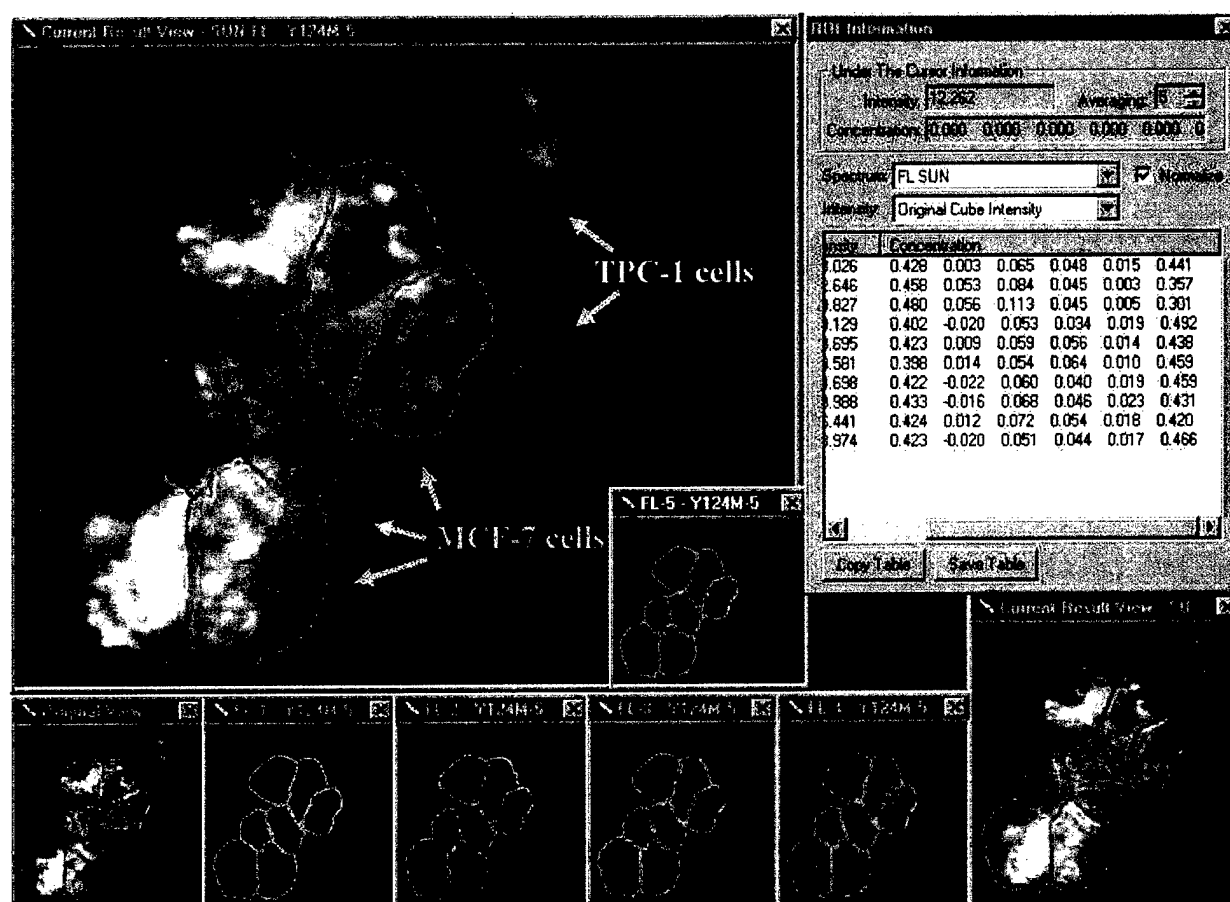
Figure 2: Gene expression analysis using Spectral Imaging. The SpectraView graphic user interface screen shows the analysis of a Spectral Image recorded from a mixture of MCF-7 and TPC-1 cells hybridized with five cDNA probes. The DAPI picture (DNA counterstain; not shown) was recorded separately. The image illustrates the process of defining regions of interest and the measurements performed by the software.

9

KEY RESEARCH ACCOMPLISHMENTS:

- Initiated search for full length cDNA clones that correspond to the 'novel' tk genes and isolated one clone
- Simplified the oligonucleotide design
- Developed a 2-step DNA amplification protocol
- Improved efficiency of in vitro DNA amplification
- Reduced template-dependant differences during DNA amplification
- Developed FISH protocols for visualization and intracellular localization of transcripts
- Developed a Spectral Imaging-based system to quantitate expression levels of 5 different genes on a cell-by-cell base

REPORTABLE OUTCOMES:

- **manuscripts**

1. Weier H-UG, Munné S, Lersch RA, Hsieh HB, Smida J, Chen XN, Korenberg JR, Pedersen RA, Fung J (2001) Towards a Full Karyotype Screening of Interphase Cells: 'FISH and Chip' Technology. Molecular and Cellular Endocrinology 183, Suppl. 1:S41-45

2. Weier, H.-U.G. (2002) Quantitative DNA Fiber Mapping. In: FISH Technology. B. Rautenstrauss and T. Liehr (Eds.), Springer Verlag, Heidelberg, pp.226-253

3. Weier H-UG, Greulich-Bode KM, Ito Y, Lersch RA, Fung J (2002) Fluorescence in situ hybridization (FISH) in cancer diagnosis and prognostication: from the cause to course of the disease. Expert Review in Molecular Diagnostics 2(2):109-119

- **poster presentations**

4. Robert A. Lersch, Lisa W. Chu, Yuko Ito and Heinz-Ulrich G. Weier. Toward Tyrosine Kinase Expression Profiling at the Single Cell Level. The Sixth Joint Meeting of the of the Japan Society of Histochemistry and Cytochemistry and the Histochemical, July 18-21, 2001, Seattle, WA.

5. Heinz-Ulrich G. Weier, Yuko Ito, Jingly Fung, Lars Lehmann, Robert A. Lersch, Lisa W. Chu, Horst F. Zitzelsberger. Chromosome rearrangements in a cell line derived from a case of childhood papillary thyroid cancer (chPTC) with radiation history. The Sixth Joint Meeting of the of the Japan Society of Histochemistry and Cytochemistry and the Histochemical, July 18-21, 2001, Seattle, WA.

6. Yuko Ito, Jingly Fung, Joanne Hsu, Nir Katzir, Robert A Lersch and Heinz-Ulrich G. Weier. Phenotype analysis of tumor cells with eight color FISH. The Sixth Joint Meeting of the of the Japan Society of Histochemistry and Cytochemistry and the Histochemical, July 18-21, 2001, Seattle, WA.

- **funding obtained**

National Institute of Health, 'Spectral Karyotyping for Phenotype Analysis of Cancer Cells', R33 Supplement, H.-U. Weier (P.I.), 9/01/01-8/31/03

CONCLUSIONS:

This IDEA project is mostly on track and has met almost all of its milestones. The soft- and hardware components necessary for these studies were put in place in the first year. The results obtained with RNA isolated from cell lines and breast tissues have proven the hypothesis that changes in tk gene expression can be monitored by a combination of PCR using tk gene family-specific primers and DNA micro-arrays. While the hybridization to the DNA micro-array appeared to possess the required specificity, second year research addressed the issues of hybridization background reduction and definition of a suitable reference DNA probe. Comparison of the cDNA micro-array data with those obtained by Southern blot analyses suggested a non-homogeneous amplification of tk fragments. This has been addressed by an altered PCR protocol involving new oligonucleotide primers. Due to a reduction in the P.I.'s effort due to a period of family medical leave, the expression profiling of small tissues samples obtained by tissue microdissection and databasing of results will be addressed in a fourth year.

REFERENCES:

None.

APPENDICES:

1.Weier H-UG, Munné S, Lersch RA, Hsieh HB, Smida J, Chen XN, Korenberg JR, Pedersen RA, Fung J (2001) Towards a Full Karyotype Screening of Interphase Cells: 'FISH and Chip' Technology. Molecular and Cellular Endocrinology 183, Suppl. 1:S41-45

2. Weier, H.-U.G. (2002) Quantitative DNA Fiber Mapping. In: FISH Technology. B. Rautenstrauss and T. Liehr (Eds.), Springer Verlag, Heidelberg, pp.226-253

3. Weier H-UG, Greulich-Bode KM, Ito Y, Lersch RA, Fung J (2002) Fluorescence in situ hybridization (FISH) in cancer diagnosis and prognostication: from the cause to course of the disease. Expert Review in Molecular Diagnostics 2(2):109-119

4. Robert A. Lersch, Lisa W. Chu, Yuko Ito and Heinz-Ulrich G. Weier. Toward Tyrosine Kinase Expression Profiling at the Single Cell Level. The Sixth Joint Meeting of the of the Japan Society of Histochemistry and Cytochemistry and the Histochemical, July 18-21, 2001, Seattle, WA.

5. Heinz-Ulrich G. Weier, Yuko Ito, Jingly Fung, Lars Lehmann, Robert A. Lersch, Lisa W. Chu, Horst F. Zitzelsberger. Chromosome rearrangements in a cell line derived from a case of childhood papillary thyroid cancer (chPTC) with radiation history. The Sixth Joint Meeting of the of the Japan Society of Histochemistry and Cytochemistry and the Histochemical, July 18-21, 2001, Seattle, WA.

6. Yuko Ito, Jingly Fung, Joanne Hsu, Nir Katzir, Robert A Lersch and Heinz-Ulrich G. Weier. Phenotype analysis of tumor cells with eight color FISH. The Sixth Joint Meeting of the of the Japan Society of Histochemistry and Cytochemistry and the Histochemical, July 18-21, 2001, Seattle, WA.

III-A.4.f.   Statement of Work

Title: Expression Profiling of Tyrosine Kinase Genes

P.I.:   Heinz-Ulrich G. Weier

Task 1. To <u>identify</u> tyrosine kinase (tk) genes expressed in normal and neoplastic breast tissues (months 1-24)

- prepare cDNAs from ten cell lines and ten frozen tissue specimens (months 1-18)
- perform RT-PCR reactions and clone PCR products in plasmids (months 3-18)
- perform pre-screening with known tk fragments, cDNA sequencing and database searches (months 5-22)
- add novel clones to the panel of expressed tk gene fragments (months 6-24)

Task 2. To <u>measure</u> tk gene expression in cell lines, normal and tumor tissues (months 3-24)

- prepare DNA microarrays carrying about 100 different sequences (months 3-24)
- optimize hybridization conditions to provide quantitative information (months 6-24)
- optimize PCR parameters for quantitative amplification of target genes (months 12-18)
- develop algorithms for array readout and comparisions between measurements (months 9-24)

Task 3. To <u>validate</u> assays for multigene expression profiling in small amounts of tissue (months 18-36)

- develop software for databasing, automated analysis of expression profile datasets and their annotation (months 18-24)
- test the system with serial dilutions of cells (months 24-30)
- test the assay with microdissected tissue from breast cancer sections (months 30-36)

Toward Tyrosine Kinase Expression Profiling at the Single Cell Level

Robert A. Lersch, Lisa W. Chu, Yuko Ito, and Heinz-Ulrich G. Weier
Department of Subcellular Structures, Life Sciences Division, University of California, E.O. Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA,

cDNA microarray technology can provide an extremely detailed RNA expression profile of tissues. To be maximally useful in research or medicine, expression profiling should be extended to individual cells. Cell-by-cell RNA profiles would allow researchers to tackle difficult issues of tissue heterogeneity. Based on our experience cloning in tyrosine kinase genes, we have developed a PCR-based method that allows us to determine the expression profile of tyrosine kinase genes in a tissue. This method relies on degenerate primers designed to amplify a region between two relatively well-conserved domains within a tyrosine kinase transcript. The primer binding sites have been selected to be approximately the same distance apart so the PCR products can be visualized and purified to greatly improve probe specificity. The amplified sequences between priming sites are sufficiently divergent to determine an expression profile by DNA microarray hybridization. Since our method relies on well-understood PCR techniques, it can be extended to single cell analysis. We are currently refining this method to make a representative tyrosine kinase profile of a single cell. We view this single cell profiling as an essential complement to our efforts in phenotypic analysis with multi-color FISH (see abstract by Ito et al.). Furthermore, since our method relies upon making degenerate primers to sequences that define a gene family, we believe that this approach is generally applicable to profile other gene families such as serine/threonine kinases, transcription factors, and other critical regulators of cellular control that have conserve domains.

Chromosome rearrangements in a cell line derived from a case of childhood papillary thyroid cancer (chPTC) with radiation history

Heinz-Ulrich G. Weier[1], Yuko Ito[1], Jingly Fung[1,2], Lars Lehmann[3], Robert A. Lersch[1], Lisa W. Chu[1] and Horst F. Zitzelsberger[4]

[1]Department of Subcellular Structures, Life Sciences Division, University of California, E.O. Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA,

[2]Reproductive Genetics Unit, Department of Obstetrics and Gynecology, University of California, San Francisco, CA 94143-0720, USA,

[3]Roche Molecular Biochemicals, Roche Diagnostics GmbH, D-82372 Penzberg, Germany and

[4]GSF-National Research Centre for Environment and Health, Institute of Molecular Radiobiology, D-85764 Oberschleißheim, Germany.

Papillary thyroid cancer occurs in several forms such as sporadic or familial as well as after exposure to ionizing radiation. Although these tumors appear to be caused by different genetic or external factors, many of them share a surprising phenotypical similarity. Few genes are known to be expressed abnormally in chPTC with radiation history. The most common rearrangement leads to abnormal expression of the catalytic domain of the ret proto-oncogene, a tyrosine kinase (tk) gene that maps to chromosome 10q11.2. Searching for other abnormally expressed genes involved in the chPTC phenotype, we applied molecular cytogenetic methods to the investigation of chromosome rearrangements in the chPTC cell line S48. This cell line forms tumors in nude mice, yet tested negative for expression of normal or rearranged copies of ret. G-banding analysis suggested complex rearrangements and the presence of several marker chromosomes. Comparative genomic hybridization indicated gains and losses of parts of several chromosomes, most notably chromosomes 1, 2, 6, 9, 13, and 22. These structural alterations were confirmed by Spectral Karyotyping which indicated only a single reciprocal translocation in the present of several more complex rearrangements. A combination of positional cloning and candidate gene approaches lead to a better characterization of chromosomal breakpoints, and the discovery of two rearranged forms of another tk gene, the tropomyosin receptor kinase NTRK1.

Phenotype analysis of tumor cells with eight color FISH

Yuko Ito[1], Jingly Fung[1,2], Joanne Hsu[1], Nir Katzir[3], Robert A Lersch[1] and Heinz-Ulrich G. Weier[1]

[1] Life Sciences Division, University of California, E.O. Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA, [2]Reproductive Genetics Unit, Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco, CA 94143-0720, USA, and [3]Applied Spectral Imaging, Migdal HaEmek 10551, Israel.

High throughput gene expression profiling using cDNA microarrays generates a wealth of information and often demonstrates tumor-specific changes. These measurements, however, provide average values for tumor cell populations that may be rather heterogeneous. Our technical developments address the issue of heterogeneity in tumor research by developing an analytical system capable of performing semi-quantitative multi-gene expression profiling of single cells. Targeting cell-by-cell measurements of expression levels of multiple tumor markers, our approach uses RNA/cDNA fluorescent in situ hybridization (FISH) combined with Spectral Imaging and digital image analysis. While the system is capable of deconvoluting images of objects stained with up to nine fluorochromes, we performed initial tests of system resolution and reproducibility with commercially available beads fluorescing in seven different wavelength intervals. The system measured up to our expectation of being able to quantitate the seven different fluorescent reporter molecules with relative standard deviations ranging from 1% to 6.1%. Using eight different fluorochromes, we then analyzed the expression levels of 6 different tyrosine kinase gene and one genomic target in breast and thyroid cancer cells counterstained with DAPI. In artificial mixtures, the system was able to recognize the tumor cells based on the level of expression of one or two genes, and could identify cells present in only a few percent.

Towards a full karyotype screening of interphase cells: 'FISH and chip' technology

Heinz-Ulli G. Weier [a,*], Santiago Munné [b], Robert A. Lersch [a], H.-Ben Hsieh [a],
Jan Smida [c], Xiao-Ning Chen [d], Julie R. Korenberg [d], Roger A. Pedersen [e],
Jingly Fung [a,e]

[a] *Department of Subcellular Structure, Life Sciences Division MS 74-157, University of California,
E.O. Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA 94720, USA*
[b] *The Institute for Reproductive Medicine and Science, Saint Barnabas Medical Center, Livingston, NJ 07052, USA*
[c] *Institute of Radiobiology, GSF-Forschungszentrum für Umwelt und Gesundheit GmbH, D 85758 Oberschleissheim, Germany*
[d] *Medical Genetics Birth Defects Center, University of California, Los Angeles, CA 90048, USA*
[e] *Reproductive Genetics Unit, Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco,
CA 94143-0720, USA*

Abstract

Numerical chromosome aberrations are incompatible with normal human development. Our laboratories develop hybridization-based screening tools that generate a maximum of cytogenetic information for each polar body or blastomere analyzed. The methods are developed considering that the abnormality might require preparation of case-specific probes and that only one or two cells will be available for diagnosis, most of which might be in the interphase stage. Furthermore, assay efficiencies have to be high, since there is typically not enough time to repeat an experiment or reconfirm a result prior to fertilization or embryo transfer. Structural alterations are delineated with breakpoint-spanning probes. When screening for numerical abnormalities, we apply a Spectral Imaging-based approach to simultaneously score as many as ten different chromosome types in individual interphase cells. Finally, DNA micro-arrays are under development to score all of the human chromosomes in a single experiment and to increase the resolution with which micro-deletions can be delineated. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cytogenetics; Chromosomes; Hybridization; FISH; DNA micro-arrays; Digital image analysis (blastomeres)

1. Introduction

Carriers of balanced translocations have an elevated risk of producing aneuploid germ cells due to disturbed homologue pairing. The resulting partial or total aneusomies lead to spontaneous abortions, stillbirth or severe deficiencies and disease. Assisted reproductive technology now offers couples at risk several diagnostic approaches to reduce the risk of carrying an affected fetus. If the woman carries the abnormality, first polar bodies can be analyzed immediately after oocyte harvest. Following in vitro fertilization, pre-implantation genetic analysis (PGD) can be performed on individual blastomeres biopsied from 3-day-old embryos. Since most of the embryonic cells will be found in interphase stage, the diagnostic approach will have to work reliably with either the less condensed chromatin in interphase cell nuclei or the highly condensed DNA in polar bodies (PB's).

Our collaborating laboratories have long been involved in the development of nucleic acid hybridization-based procedures for the rapid detection of structural and numerical chromosome abnormalities. Here, we report the present state of hybridization-based technologies for interphase cell analysis in PGD. Since only one or two cells are available for analysis, our approaches are geared towards obtaining a maximum of cytogenetic information per experiment.

* Corresponding author. Tel.: +1-510-486-5347; fax: +1-510-486-5343.

E-mail address: ugweier@lbl.gov (H.-U.G. Weier).

2. Material and methods

For more than a decade, our laboratories have been involved in the development of technologies for analysis of interphase and metaphase cells. In collaboration with scientists at the University of California, San Francisco, and the St. Barnabas Medical Center, Livingston, researchers at the E.O. Lawrence Berkeley National Laboratory study the chromosomal composition of blastomeres with regard to numerical as well as structural aberrations. The technical aspects of our probe preparation and multicolor detection protocols have been published previously (Weier et al., 1994; Jossart et al., 1996; Cassel et al., 1997).

A major goal of our technical developments is to maximize the number of chromosomal targets that can be scored simultaneously. Briefly, probes specific for repeated DNA on chromosomes 15, X, and Y purchased from Vysis (Downers Grove, IL) were labeled with either a green or red fluorochrome (Spectrum Green or Spectrum Orange, respectively). The probes specific for chromosome 9, 13, 14, 16, 18, 21, and 22 were prepared in-house and labeled by random priming (BioPrime Kit, GIBCO/LTI, Gaithersburg, MD) incorporating biotin-14-dCTP (part of the BioPrime Kit), digoxigenin-11-dUTP (Roche Molecular Biochemicals, Indianapolis, IN), fluorescein-12-dUTP (Roche Molecular Biochemicals) (Weier et al., 1995), or Cy3-dUTP (Amersham, Arlington Heights, IN). Bound biotinylated probes were detected with avidin-Cy5, and bound digoxigenin-labeled probes were detected with Cy5.5-conjugated antibodies against digoxin (Sigma, St. Louis, MO). Between 0.5 and 3 µl of each probe along with 1 µl human COT1™ DNA (1 mg/ml, GIBCO/LTI) and 1 µl salmon sperm DNA (20 mg/ml, 3′–5′, Boulder, CO) were precipitated with 1 µl glycogen (Roche Molecular Biochemicals, 1 mg/ml) and 1/10 volume of 3 M sodium acetate in 2 volumes of 2-propanol, air dried and resuspended in 3 µl water, before 7 µl of hybridization master mix [78.6% formamide (FA, GIBCO/LTI), 14.3% dextran sulfate in 2.9 × SSC, pH 7.0 (1 × SSC is 150 mM NaCl, 15 mM Na citrate)] were added. This gave a total hybridization mixture of 10 µl.

All blastomeres used in the probe developments were obtained from embryos donated by patients enrolled in the IVF Programs of The University of California, San Francisco, or The Institute for Reproductive Medicine and Science of Saint Barnabas Medical Center. In accordance with guidelines set by the internal review boards of these Medical Centers, written consent was obtained from the patients in each case. Embryo biopsies and blastomere fixations were carried out as described (Munné et al., 1994, 1996). As indicated below, embryos used for some studies had arrested development or were morphologically abnormal.

3. Results and discussion

Traditional filter based microscope systems limited fluorescence in situ hybridization (FISH) experiments to the simultaneous use of typically no more than three to five differently labeled probes for interphase analysis (Munné and Weier, 1996). This is sufficient to detect structural alterations in interphase and metaphase cells or score a few chromosomes in interphase cells (Munné et al., 1994; Munné and Weier, 1996; Munné et al., 1996). We prepared case-specific breakpoint-spanning probe contigs to identify intrachromosomal rearrangements such as inversions or deletions (Cassel et al., 1997). The same approach can be used to demonstrate interchromosomal rearrangements such as reciprocal translocations (Munné et al., 1998a; Fung et al., 1998a, 1999; Weier et al., 1999). The case-specific probes allow one to discriminate between a normal karyotype, aneuploid cells, and a balanced karyotype carrying the derivative chromosomes. A less time consuming and, thus, less expensive approach using DNA probes that bind distal to the respective breakpoints can only be used to count the number of chromosome copies and thus cannot discriminate between the normal and the balanced karyotypes (Munné et al., 1998b).

3.1. Detection of structural chromosome aberrations

Our scheme for the detection of structural alteration is based on the preparation and hybridization of two differently labeled DNA probes that bind on both sides of the respective chromosome breakpoints (Cassel et al., 1997). One probe will be detected in the green fluorescence wavelength interval, while the second probe is made such that it fluoresces red. Normal homologues lack the rearrangement and the probes produce large hybridization domains that appear either red or green in the fluorescence microscope. At the same time, we counterstain the DNA with 4,6-diamino-2-phenylindole (DAPI) which fluoresces blue under ultraviolet light excitation. If the cell contains a derivative chromosome, the hybridization result will show a red/green associated or partially overlapping signal indicative of the translocation event. Thus, we detect structural alteration and score homologues at the same time.

Our work is greatly facilitated by access to resources created in the course of the International Human Genome Sequencing Project, such as large insert genomic DNA libraries (bacterial or yeast artificial chromosomes (BACs or YACs, respectively), high resolution physical maps or collections of cytogenetically mapped DNA probes (Chen et al., 1996; Kim et al., 1996; Korenberg et al., 1999). This enables us to prepare case-specific probe sets suitable for interphase cell analyses of most patient cells within a few weeks.

Once optimized, these probe sets allow to rapidly determine the exact number of normal chromosomes and derivative chromosomes in somatic cells from translocation carriers as well as their germ cells or offspring. So far, however, these procedures failed to produce the desired increase in pregnancy rates in cases where one spouse carried a balanced reciprocal translocation. Our concern is that the impaired homologue pairing in the carriers leads to gain or loss of other chromosomes which remains undetected in assays scoring only the translocation chromosomes.

The recent introduction of Spectral Imaging (SIm) now allows one to interrogate a much larger number of targets, thus producing a more comprehensive picture of the chromosomal composition of the cells. SIm allows an investigator to discriminate an increased number of fluorescent probes by exciting fluorescent molecules over a broad spectral range and by recording the fluorescence emission spectral using an interferometer.

3.2. Detection of numerical chromosome abnormalities using Spectral Imaging (SIm)

Chromosome abnormalities occur with astonishing frequency in humans, being present in an estimated 10–30% of all fertilized eggs. Over 25% of all the miscarriages are monosomic or trisomics, making aneuploidy the leading known cause of pregnancy loss. Ideally, one likes to detect aneuploidy involving any of the 24 human chromosomes for preimplanation genetic and prenatal diagnosis. Thus, an analytical method to enumerate as many chromosomes as possible in few interphase cells is highly desirable. Using a set comprised of seven chromosome-specific probes (chromosome 10, 14, 16, 18, 22, X and Y) hybridized to lymphocyte interphase nuclei, we demonstrated that Spectral Imaging system provides a significant improvement over conventional filter-base microscope systems for enumeration of multiple chromosomes in interphase nuclei (Fung et al., 1998b).

Using mostly yeast or bacterial artificial chromosome probes for cytogenetic analyses of blastomeres and detection of structural alterations, we are building panels of probes to simultaneously score 10 or more different chromosomes. Further increases in the number of probes is complicated due to occasional overlap of chromosome domains or local variation in hybridization efficiency. We developed a 10-chromosomes probe set (chromosomes 9, 13, 14, 15, 16, 18, 21, 22, X and Y) for the purpose of labeling DNA targets most frequently associated with aneuploidy and spontaneous abortions and tested its application in PGD (blastomeres from abnormal human preimplantation embryos) and prenatal diagnosis (uncultured amniocytes obtained by amniocentesis). Results demonstrated in-

creasing levels of background fluorescence on different cells after hybridization in the order: (uncultured amniocytes) ≫ (blastomeres) > (interphase cells from lymphocytes). All blastomeres fixed for this study (*N* = 25) spread very well. Fourteen nuclei (56%) showed interpretable hybridization results, and most of them were karyotyped as abnormal, since all those cells were from either 1 pronucleus (PN) or 3 PN human embryos, and had arrested development or were morphologically abnormal. The signals from 11 nuclei (44%) were faint. This may be related to the quality of the embryos, since all of them were developing abnormally. The fixation of uncultured amniocytes on slides for Spectral Imaging analysis turned out to be somewhat difficult. Most nuclei were not flattened out, presenting a problem due to the limited focal depth. Overlapping signal domains were a problem in uncultured amniocytes, where only about 20% of all cells showed interpretable spreads. In summary, Spectral Imaging has demonstrated advantages for evaluating numerical chromosomal abnormalies in single interphase cells. Its utility for chromosome scoring, however, remains limited due to chromosome domain overlap.

3.3. DNA micro-arrays (chips)

The DNA micro-arrays represent an exciting new technology with applications ranging from gene expression profiling to determination of gene copy number changes in tumors. We are presently investigating this approach in which the DNA probes are immobilized on glass slides as a strategy complementing FISH studies. The DNA from the embryonic cells is labeled in one color (e.g. red), while an equal amount of a reference DNA probe is labeled in a different color (e.g. green). These differentially labeled DNA are combined, denatured and hybridized to a DNA micro-array or 'chip' in a quantitative manner. Results are obtained after reading the micro-arrays with specially designed fluorescence scanners as red/green or red/infrared fluorescence ratios. After normalization, every increase or decrease from the average ratio indicates an abnormal number of copies of the hybridization target.

In practice, the DNA contained in a single cell is not sufficient to generate measurable signals. The commonly used protocols therefore include a DNA in vitro amplification step using a random primer or oligonucleotide with arbitrary sequence prior to labelling. The DNA to be immobilized can be obtained by standard isolation protocols or by in vitro DNA amplification. We use a DNA spotter based on the design published by Brown's group at Stanford University (Schena et al., 1996). This allows us to spot small amounts of DNA on poly-L-lysine coated glass slides with a 100–200 micron pitch. A 288-spot DNA micro-array like the test array depicted schematically in Fig. 1, then measures no more

than a few square millimeters. This small size of micro-arrays is an advantage over larger arrays prepared on nylon filters, because it requires less amount of labeled DNA sample in the hybridization reaction.

We are presently preparing DNA test chips to develop the methods and study parameters such as probe preparation, hybridization conditions and chip reader performance. Our long term objective is the development of reliable procedures to detect structural as well as numerical abnormalities using a combination of 'FISH and chip' technology. Chips to be used in those studies will carry several hybridization targets per chromosome arm, thus allowing a more detailed gene dosage determination or delineation of full or partial aneusomies than the FISH-based assays.
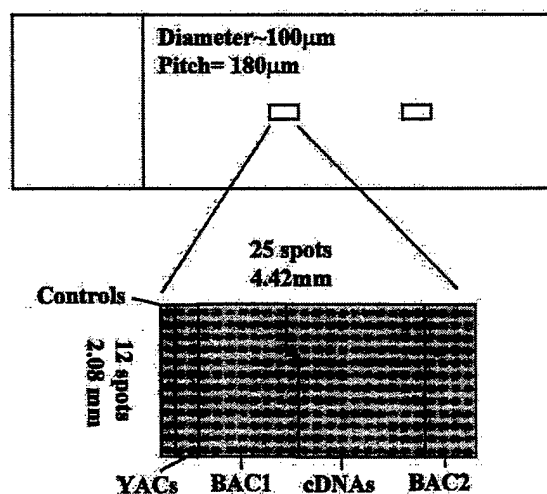
Fig. 1. Prototype DNA micro-array for technology development. The chip contains from left to right: control DNA spots (six samples), PCR amplified YAC DNA (12 clones), BAC array 1 (46 clones for copy number determination in breast cancer research), cDNA (62 tyrosine kinase cDNA for expression profiling), and BAC array 2 (24 clones for chromosome enumeration in PGD). All samples are spotted in duplicate. The total number of DNA spots of 300 arrayed with a 180 μm pitch leads to array dimensions of 2.08 × 4.42 mm². Each slide contains two identical micro-arrays.

References

Cassel, M.J., Munné, S., Fung, J., Weier, H.U.G., 1997. Carrier-specific breakpoint-spanning DNA probes: an approach to pre-implantation genetic diagnosis [PGD] in interphase cells. Hum. Reprod. 12, 101–109.

Chen, X.-N., Mitchell, S., Sun, Z.G., Noya, D., Ma, S., Sekhon, G.S., Thompson, K., Hsu, W.-T., Wong, P., Wang, N., Shcreck, R., Falk, R., Korenberg, J.R., 1996. The Integrated BAC Resource: a powerful new molecular tool for prenatal genetics. Am. J. Hum. Genet. 59, A12.

Fung, J., Munné, S., Duell, T., Weier, H.-U.G., 1998a. Rapid cloning of translocation breakpoints: from blood to YAC in 50 days. J. Biochem. Mol. Biol. Biophys. 1, 181–192.

Fung, J., Hyun, W., Dandekar, P., Pedersen, R.A., Weier, H.-U.G., 1998b. Spectral imaging in preconception/preimplantation genetic diagnosis (PGD) of aneuploidy: multi-colour, multi-chromosome screening of single cells. J. Ass. Reprod. Genet. 15, 322–329.

Fung, J., Munné, J., Garcia, J., Kim, U.-J., Weier, H.-U.G., 1999. Reciprocal translocations and infertility: molecular cloning of breakpoints in a case of constitutional translocation t(11;22)(q23;q11) and preparation of probes for preimplantation genetic diagnosis (PGD). Reprod. Fert. Devel. 11, 17–23.

Jossart, G.H., O'Brien, B., Cheng, J.-F., Tong, Q., Jhiang, S.M., Duh, Q., Clark, O.H., Weier, H.-U.G., 1996. A novel multicolor hybridization scheme applied to localization of a transcribed sequence (D10S170/H4) and deletion mapping in the thyroid cancer cell line TPC-1. Cytogenet. Cell Genet. 75, 254–257.

Kim, U.J., Shizuya, H., Kang, H.L., Choi, S.S., Garrett, C.L., Smink, L.J., Birren, B.W., Korenberg, J.R., Dunham, I., Simon, M.I., 1996. A bacterial artificial chromosome-based framework contig map of human chromosome 22q. Proc. Natl. Acad. Sci. USA 93, 6297–6300.

Korenberg, J.R., Chen, X.-N., Sun, Z., Shi, Z.-Y., Shi, Ma.S., Vataru, E., Yimlamai, D., Weissenbach, J.S., Shizuya, H., Melvin, S.I., Gerety, S.S., Nguyen, H., Zemsteva, I.S., Hui, L., Silva, J., Wu, X., Birren, B.W., Hudson, T.J., 1999. Human Genome Anatomy: BACs integrating the genetic and cytogenetic maps for bridging genome and biomedicine. Genome Res. 9, 994–1001.

Munné, S., Grifo, J., Cohen, J., Weier, H.-U.G., 1994. Mosaicism and aneuploidy in arrested human preimplantation embryos: a multiple probe fluorescence in situ hybridization (FISH) study. Am. J. Hum. Genet. 55, 150–159.

Munné, S., Dailey, T., Finkelstein, M., Weier, H.U.G., 1996. Reduction in overlaps and missing signals in interphase-FISH. J. Assist. Reprod. Genet. 13, 149–156.

Munné, S., Weier, H.-U.G., 1996. Simultaneous enumeration of chromosomes X, Y, 18, 13, and 21 in interphase cells for preimplantation genetic diagnosis of aneuploidy. Cytogenet. Cell Genet. 75, 264–270.

Munné, S., Fung, J., Cassel, M.J., Márquez, C., Weier, H.-U.G., 1998a. Preimplantation genetic analysis of translocations: case-specific probes for interphase cell analysis. Hum. Genet. 102, 663–674.

Munné, S., Morrison, L., Fung, J., Márquez, C., Weier, U., Bahçe, M., Sable, D., Grundfelt, L., Schoolcraft, B., Scott, R., Cohen, J., 1998b. Spontaneous abortions are significantly reduced after preconception genetic diagnosis of translocations. J. Ass. Reprod. Genet. 15, 290–296.

Schena, M., Shalon, D., Heller, R., Chai, A., Brown, P.O., Davis, R.W., 1996. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. Proc. Natl. Acad. Sci. USA 93, 10614–10619.

Weier, H.-U., Rosette, C.D., Matsuta, M., Zitzelsberger, H., Matsuta, M., Gray, J., 1994. Generation of highly specific DNA hybridization probes for chromosome enumeration in human interphase cell nuclei: isolation and enzymatic synthesis of alpha satellite DNA probes for chromosome 10 by primer directed DNA amplification. Meth. Mol. Cell Biol. 4, 231–248.

Weier, H.-U.G., Wang, M., Mullikin, J.C., Zhu, Y., Cheng, J.F., Greulich, K.M., Bensimon, A., Gray, J.W., 1995. Quantitative DNA fiber mapping. Hum. Mol. Genet. 4, 1903–1910.

Weier, H.-U.G., Munné, S., Fung, J., 1999. Patient-specific probes for preimplantation genetic diagnosis (PGD) of structural and numerical aberrations in interphase cells. J. Assist. Reprod. Genet. 16, 182–189.

Quantitative DNA Fiber Mapping

HEINZ-ULRICH G. WEIER

Introduction

High resolution physical maps have become indispensable for the positional cloning of disease genes and large scale sequencing projects. Common maps are based on ordered sets of clones from sources such as cosmid, P1/PAC/ BAC, or yeast artificial chromosome (YAC) libraries. The assembly of such maps is facilitated by application of fluorescence in situ hybridization (FISH). Hybridization of non-isotopically labeled probes onto preparations of DNA molecules ('DNA fibers') that were bound with one or both ends to a solid substrate and stretched homogeneously, forms the base of our 'Quantitative DNA Fiber Mapping (QDFM)' technique. Because the DNA fibers are easily accessible to probes and detection reagents, hybridization efficiencies are typically high and allow DNA targets as small as 500-1000 bp to be detected routinely. Quantitative DNA fiber mapping experiments require only standard laboratory equipment and access to a fluorescence microscope. By hybridizing one clone onto another, the extent and orientation of overlaps can be quantitated with near kilobase resolution. To measure the physical distance between non-overlapping DNA fragments, probes are hybridized to DNA fibers representing a larger genomic interval (Fig. 1). This also allows the mapping of expressed sequences (cDNAs) along DNA fibers representing genomic DNA.

Heinz-Ulrich G. Weier, University of California, E.O. Lawrence Berkeley National Laboratory, Department of Subcellular Structure, Life Sciences Division, M.S. 74-157, 1 Cyclotron Road, Berkeley, CA, 94720, USA (*phone* +510-486-5347; *fax* +510-486-5343; *e-mail* ugweier@lbl.gov)
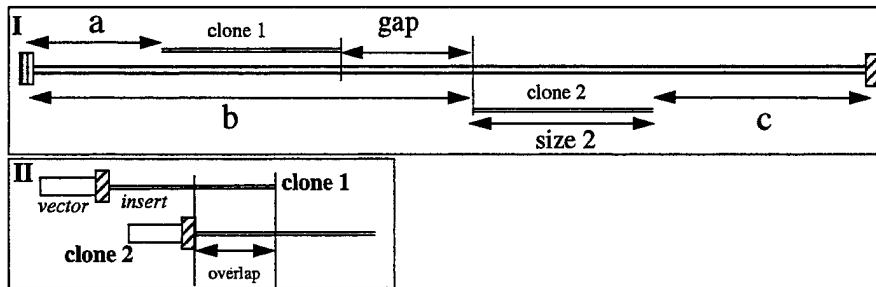
Fig. 1. Physical mapping strategies. I Mapping a small DNA molecule on to a larger molecule; II mapping of clone overlap by pairwise hybridization

Outline

- Isolate DNA

- Pretreat microscope slides or coverslips

- Prepare DNA fibers on glass

- Select probe set

- Denature and hybridize probe set

- Remove unbound probe

- Detect bound probes with antibodies

- Acquire images

- Analyze images

- Save results in spreadsheets for further analysis

Principles and applications

High resolution physical maps have proven indispensable for large-scale, cost-effective gene discovery. Knowledge about the extent of overlap between any two clones and the precise localization of cloned DNA fragments within much larger genomic fragments is needed to assemble such maps. As demonstrated in this chapter, FISH can provide this critical information.

Isolation of DNA from cell nuclei and preparation of some sort of chromatin 'fibers' improves the accessibility of the DNA targets for probes as

Materials

- Refrigerated Centrifuge (MP4R, IEC)   **Equipment**
- Dry bath (Model 2001, Labline Instruments)
- Fluorescence microscope (Axioskop, Zeiss) equipped with 40× and 63× oil immersion lenses
- Incubator oven (set to 37°C, Precision Scientific)
- Pulsed Field Gel Electrophoresis (PFGE) system (Biorad)
- Shaking Incubators (New Brunswick): 30°C for yeast cell culture, 37°C for culture of *E. coli*
- Thermal cycler for in vitro DNA amplification
- Water bath (Model 188, Precision Scientific)
- Digital Imaging system (optional)

- 3-Aminopropyltriethoxy silane (APS, Sigma Chemicals)   **Reagents**
- β-Mercaptoethanol (Sigma Chemicals)
- β-Agarase (New England Biolabs (NEB))
- Agarose (Life Technologies)
- Antibodies against digoxigenin, rhodamine-conjugated, made in sheep (Roche Molecular Biochemicals), stock solution is 1 mg/ml in PNM, dilute 1:50 with PNM prior to use. Store at 4°C.
- Antibodies against FITC, made in mouse (DAKO), stock solution is 1 mg/ml in PNM, dilute 1:50 prior to use. Store at 4°C.
- Anti-mouse antibodies, FITC conjugated, made in horse (Vector Labs), stock solution is 1 mg/ml in PNM, dilute 1:50 prior to use. Store at 4°C.
- Anti-avidin antibodies, biotinylated, made in goat (Vector Labs), stock solution is 1 mg/ml in PNM, dilute 1:50 prior to use. Store at 4°C.
- Avidin conjugated to AMCA (Vector Labs), stock solution is 2 mg/ml in PNM, dilute 1:500 prior to use. Store at 4°C.
- Blocking reagent: cat. #1096-176 (Roche Molecular Biochemicals)
- Chloroform/isoamyl alcohol: 24:1 vol:vol. (Life Technologies)

- RNase (Roche Molecular Biochemicals), DNase-free: boil at 100°C for 10 min., aliquot and store at -20°C

- Salmon sperm DNA (3'-5', Boulder, CO), 20 mg/ml. Store at -20°C.

- Sodium dodecyl sulfate (SDS)(Na salt, Sigma Chemicals): 10% in water.

- Thermus aquaticus (Tag) DNA polymerase, 5 U/μl (Perkin Elmer). Store at -20°C.

- Ultrapure water (Mallinckrodt, cat. # H453)

- Yeast artificial chromosome (YAC) library (Research Genetics). Store at -80°C.

- YOYO-1 (Molecular Probes): Stock is 1mM in DMSO. Dilute 1:1000 with water prior to use. Store at -20°C and discard diluted dye after 1 week.

- Zymolase (Sigma Chemicals, 70,000 U/g): prepare 10 mg/ml in 50 mM KH_2PO_4, pH 7.8, 50% glycerol. Store at -20°C.

- AHC medium (BIO 101): add 36.7 g of AHC powder per liter of purified water, autoclave at 121°C for 15 min.

Buffers and other solutions

- AHC agar (BIO 101): add 53.7 g of AHC agar medium per liter of purified water. Autoclave at 121°C for 15 min. Cool to 50°C. Then, mix well and pour plates. Store plates at 4°C.

- Alkaline Lysis (AL) solutions sufficient for 12 preps at the level of 20 ml cell culture:
    - AL Solution I: 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0, add 4 ml of 0.5 M glucose, 0.8 ml of 0.5 M EDTA and 1 ml of 1 M Tris-HCl to 34.2 ml water. Store at 4°C.
    - AL Solution II: 0.2 N NaOH, 1% SDS. Add 1.4 ml of 10 N NaOH, 7 ml of 10% SDS to 61.6 ml water.
    - AL Solution III: 3 M NaOAc, pH 4.8

- Antifade solution: 1% p-phenylenediamine (Sigma), 15 mM NaCl, 1 mM H_2PO_4, pH 8.0, 90% glycerol. Store at -80°C.

- Blocking stock solution: dissolve blocking reagent (Roche Molecular Biochemicals, cat. # 1096 176) in maleic acid buffer (10% w/v) with shaking and heating. Autoclave stock solution and store in aliquots at 4°C.

- 10× Taq buffer: 500 mM KCl, 100 mM Tris HCl, pH 8.3, 10 mM MgCl$_2$.

- TBE (Tris/borate/EDTA) buffer, 10× is 890 mM Tris base, 890 mM boric acid, 20 mM EDTA.

- TE (Tris/EDTA) buffer, 1× is 10 mM Tris HCl, 1 mM EDTA, pH 7.4, 7.5 or 8.0.

- TE 50 buffer: 10 mM Tris HCl, 50 mM EDTA, pH 7.8.

- Tris HCl [tris(hydroxymethyl)aminomethane]: 1 M, pH 7.5 or 8.0.

Subprotocol 1
Preparation of aminopropyltriethoxysilane (APS) derivatized slides

The derivatization of glass substrates is among the most critical steps of the procedure. The slides should have the capacity to bind DNA molecules at one or both ends, but allow the molecules to stretch during the subsequent drying.

Procedure

1. Slide preparation

1. Clean glass slides mechanically by repeated rubbing with wet cheesecloth to remove dust and glass particles.

2. Rinse several times with ultrapure water.

3. Immerse slides in boiling ultrapure water for 10 min.

4. Air dry.

5. Immerse slides in 18 M sulfuric acid for at least 30 min to remove organic residues.

6. Immerse in boiling water for 1-2 min.

7. Air dry and store until further use.

The DNA is recovered from the low melting point agarose slab gel by excising the appropriate band using a knife or razor blade. High molecular weight DNA is then isolated by β-agarase digestion of the gel slices.

Procedure

1. Pulsed field gel electrophoresis (PFGE)

1. Spin down cells from 5 ml AHC media at 400 rpm for 6 min. Resuspend cells in 0.5 ml of 0.125 M EDTA, pH 7.8. Spin again and remove super-natant.

2. Resuspend the ~ 70 μl cell pellet in 500 μl of SCE. Mix with an equal volume of 1.5% LMP agarose preheated to 43°C. Quickly pipette up and down, then vortex for 1-2 s to mix. Pipette into plug molds (Biorad) and allow to solidify at room temperature or on ice.

3. Remove plugs from molds, incubate samples in 2 ml SCE containing 100 μl of zymolase and shake at 150 rpm at 30°C for 2.5 h to overnight.

4. Remove SCE and add 2 ml of ES containing 100 μl of proteinase K (20 mg/ml). Shake 5 h to overnight at 50°C.

5. Remove ES and rinse 5 times with 6 ml of TE50 for 30 min each rinse. Store the plugs at 4°C.

Preparation of gel plugs containing YACs

1. YACs: voltage gradient, 6 V/cm; switching time, 79 s initially, 94 s final; running time, 38 h; agarose concentration, 1.0% LMP agarose; running temperature, 14°C; running buffer, 0.5×TBE.

2. P1/PAC/BAC clones: voltage gradient, 6 V/cm; switching time, 2 s initially, 12 s final; running time, 18 h; agarose concentration, 1.0% LMP agarose; running temperature, 14°C; running buffer, 0.5×TBE.

PFGE running conditions

For probe production and determination of optimal PFGE conditions: stain the gel with ethidium bromide (EB, 0.5 μg/ml in water), cut out a gel slice containing the target DNA band and transfer slice to a 14 ml poly-styrene tube (Cat.# AS-2264, Applied Scientific). Wash slice with ultrapure water for 30 min, and then wash with 1× agarase buffer for 30 min.

For high molecular weight DNA isolation: run duplicate samples on the right and left side of the gel, respectively. After a predetermined run time, cut gel in half, and stain one half with EB. Measure the migrated distance

determined primarily by the DNA preparation and by properties of the modified surface rather than by the method of DNA stretching. In general, the ideal APS-surface binds the DNA molecules only at their ends, or in the case of circular DNA molecules, at the position of nicks. The remainder of the DNA molecule should be free in suspension. This can be observed in the fluorescence microscope after addition of 1 μM YOYO-1 to the DNA before immobilization.

Procedure

In a typical experiment, 1-2 μl of clonal or genomic DNA are mixed with an equal amount of YOYO-1 (1 μM or 0.1 μM) and 8 μl water. One or two microliter of this diluted DNA is applied to an untreated coverslip, which is then placed DNA side down on the APS-treated slide or coverslip. The DNA concentration can be estimated under the fluorescence microscope using a filter set for FITC, and adjusted as needed. As early as 2 min of incubation at room temperature, the untreated coverslip can be removed slowly from one end, allowing the receding meniscus to stretch the bound DNA molecules ('fibers') in one direction (Hu et al., 1996). Alternatively, the slide or coverslip sandwich can be allowed to dry overnight at room temperature, after which the untreated coverslip is removed. Slides or coverslips carrying DNA fibers are rinsed briefly with water, drained, allowed to dry at room temperature and 'aged' in ambient air at 20°C for 1 week before hybridization.

Subprotocol 4
Probes generated from cloned DNA fragments

A typical QDFM experiment uses several different probes simultaneously. One probe is needed to counterstain the DNA fibers. This probe is usually prepared by labeling DNA from the same batch that was used to prepare the fibers. Probes for sequences to be mapped along the fibers are made such that they can be detected in a different color. Furthermore, it is recommended to include landmark probes that provide reference points by binding specifically to the vector part or the ends of DNA molecules.

13. Remove most of the bottom layer and spin again for 3 min.

14. Transfer the top layer to new microcentrifuge tubes and add 400 μl chloroform/isoamyl alcohol (24:1, vol.vol.)

15. Vortex well for 15 s, spin down for 3 min and remove most of the bottom layer followed by a second centrifugation for 3 min.

16. Transfer top layer to a new microcentrifuge tube, add 2.5 volumes, i.e., 1 ml 100% ethanol and let the DNA precipitate for 30 min at -20°C.

17. Spin down for 15 min, discard the supernatant and wash the pellet in ice cold 70% ethanol, spin again briefly, remove supernatant and air dry the pellet.

18. Resuspend the pellet in 20-40 μl TE, pH 7.4 containing 10 μg/ml RNase.

19. Incubate 30 min at 37°C [in water bath]; then, store at -20°C until used.

2. Preparation of DNA from Yeast Artificial Chromosome (YAC) clones

Retrieve the desired yeast clone containing the YAC from the library and grow it on AHC agar for 2-3 days at 30°C. Pick colonies from the plate and culture the clones in up to 35 ml AHC media at 30°C for 2-3 days.

1. Centrifuge cells (in ~35 ml AHC media) at 2000g at 4°C for 5 min.

   DNA extraction, phenol purification and alcohol precipitation

2. Decant the supernatant and resuspend cells in 3 ml total of 0.9 M sorbitol, 0.1M EDTA, pH 7.5, containing 4 μl β-mercaptoethanol, followed by addition of 100 μl of zymolase (2.5 mg/ml), and then incubate at 37°C for 60 min.

3. Pellet the cells at 2000 g and 4°C for 5 min and decant supernatant.

4. Resuspend pellet in 5 ml of 50 mM Tris, pH 7.4, 20 mM EDTA. Add 0.5 ml of 10% SDS and mix gently. Incubate at 65°C for 30 min.

5. Add 1.5 ml of 5 M potassium acetate and place on ice for 60 min.

6. Spin at 12,000 g for 15 min at 4°C, and transfer the supernatant to a new tube.

7. Mix the supernatant gently with 2 volumes of 100% ethanol by inverting the tube a few times. Spin at 5000 rpm (2000g) for 15 min at room temperature.

Procedure

1. Cloning vector-specific probes

The generation of P1/PAC-, BAC- and YAC-vector probe DNA takes advantage of the access to published vector sequences. PCR primers are typically designed to amplify fragments of 1100-1400 bp of vector sequence. Several such oligonucleotide pairs have been designed in several laboratories including ours and are used in either single pairs or combinations. The PCR usually follows standard conditions, i.e., a Tris-HCl buffer containing 1.5 mM $MgCl_2$ and 1 unit Taq DNA polymerase per 50 µl reaction is used, annealing temperatures range from 50°C to 60°C.

On the other hand, the YAC cloning vectors pJs97 and pJs98, cloned in plasmid vectors (BRL), can be used to prepare probes useful to determine the orientation of the YAC insert (Duell et al., 1997). For this purpose, plasmid DNA is extracted using the above alkaline lysis protocol or a commercial kit and labeled by random priming as described below.

2. Mixed base oligonucleotide primed PCR

The DNA probes for counterstaining the YAC DNA fibers are generated by mixed base oligonucleotide primed PCR (sometimes referred to as degenerate oligonucleotide primed PCR or 'DOP-PCR') (Cassel et al., 1997). An aliquot of the HMW DNA obtained by PFGE for fiber preparation is PCR amplified for a total of 42 cycles with oligonucleotide primers that anneal about every 200-800 nucleotides. In our preferred scheme, we use two different DNA amplification programs. Initially we perform a few manual PCR cycles using T7 DNA polymerase to extend the oligonucleotide primers at a relatively low temperature. Next, DNA copies prepared in these first cycles are amplified using the thermostable Taq DNA polymerase and a rapid thermal cycling scheme.

In the first amplification stage, T7 DNA polymerase ('Sequenase II', Amersham Pharmacia Biotech) is used in 5-7 cycles to extend the mixed base primer JUN1 (5-CCAAGCTTGCATGCGAATTCNNNNCAGG-3, N=ACGT) that is annealed at low temperature. Briefly, 2-3 µl of HMW DNA solution are removed from the bottom of each tube and PCR amplified using the following conditions: denaturation at 92°C for 3 min, primer annealing at 20°C for 2 min and extension at 37°C for 6 min. Sequenase must be added after each denaturation. (See Chapter 29 by Fung et al. for details.)

3. For labeling with either dig-dUTP or FITC-dUTP, add:
   - 2.5 µl 10× Modified Nucleotide Mixture
   - 3.25 µl 1 mM dTTP
   - 1.75 µl dig-11-dUTP or FITC-12-dUTP (1 mM, ROCHE MOLECU-
     LAR BIOCHEMICALS, #1093 088)
   - 10 µl 2.5× Random primers (BioPrime kit, Life Technologies part#
     YO1393)

   Note: For labeling the DNA with biotin, add 2.5 µl 10× dNTP mix pro-
   vided with the BioPrime kit (containing biotin-14-dCTP), 5 µl water,
   and 10 µl 2.5× random primers).

4. Mix well, add 0.5 µl DNA polymerase I Klenow fragment (40 units/µl,
   Life Technologies, part #YO1396) and incubate in a water bath at 37°C
   for 60-120 min.

5. Add 2.5 µl of 10× stop buffer (Life Technologies, part #YO1107, part of
   the BioPrime kit).

6. Store probe at -20°C until used.

Subprotocol 7
Fluorescence in situ hybridization (FISH)

All hybridizations are carried out overnight at 37°C in a moist chamber.
Fiber hybridizations include a comparatively low concentration of a bio-
tin- or FITC-labeled DNA probe prepared from the high molecular weight
DNA that is used to prepare the fibers. This counterstain highlights the
otherwise invisible DNA fibers and allows competitive displacement by
the probes to be mapped along the DNA fiber (Weier et al. 1995, Duell
et al. 1997). Additionally, one or several cloning vector-specific probes
are included to allow a determination of the orientation of the insert.

Procedure

The hybridization procedure is very similar to protocols used with meta-
phase spreads:

1. Hybridization mix: combine 1 µl of each probe, 1 µl of human COT1
   DNA (optional), 1 µl of salmon or herring sperm DNA, and 7 µl of
   hybridization master mix.

formed on images recorded on film and either printed or projected on a screen.

Procedure

Images are acquired using a standard fluorescence microscope (Zeiss Axioskop) equipped with 63×, 1.25 N.A. and 40×, 1.2 N.A. objectives, and a filter set for excitation and simultaneous observation of DAPI, Texas Red/rhodamine, FITC and CY5 fluorescence, respectively (Chroma-Technology). Current filters are capable of excitation in single bands centered around 360, 405, 490, 555, and 637 nm, and visualization in multiple bands in the vicinities of 460 nm (blue), 520 nm (green), 600 nm (red) and 680 nm (infrared). Images are collected using a CCD camera (Xilix, Hamamatsu or Photometrics) connected to a computer workstation (Weier et al., 1995).

For determination of map positions, interactive software is available for either Apple Macintosh, IBM/PC or SUN computers that allows the user to trace DNA fibers by drawing a segmented line and then calculates the length of the line in pixels (Wang et al. 1996; Duell et al. 1997). The pixel spacing is known from the microscope objective used in the experiment (use a 63× objective for molecules up to 100 kb, a 40× objective for larger molecules) and is converted into μm (or kb using the factor of 2.3 kb/μm). After measuring all relevant distances along the DNA fibers in triplicate, the results in the form of lists are imported into Microsoft Excel spreadsheets and used to calculate average values for each fiber and mean values and standard deviations for individual experiments.

Results

Solid substrates for QDFM are prepared in batches of 20-50 by derivatization of standard microscope slides, coverslips or sheets of mica with APS, which results in primary amino groups on the surface (Weier et al. 1995; Hu et al. 1996). For DNA fiber stretching, a solution of target DNA molecules onto which probes are to be mapped is placed on an untreated coverslip and spread by placing the coverslip upside-down on the APS-derivatized glass or mica surface. Binding of DNA to the substrate and the stretching effect can be monitored by staining the DNA with YOYO-1 prior to deposition. This also allows the rejection of batches of slides that bind DNA too tightly. Following DNA binding and stretch-

Preparation and purification of the circular DNA molecules is simple and fast. An example of mapping an exon specific plasmid clone onto circular BAC molecules is depicted in Fig. 3C. The use of circular DNA molecules results in a dense deposition of circular DNA molecules in the presence of linear fragments of different sizes (Fig. 3B). The largest circles are stretched to about ~2.3 kb/μm, but smaller, more condensed molecules can also be analyzed using the extent of the vector-specific green ~7 kb domain on BACs as standard for normalization. The linear fragments

Fig. 2. Quantitative DNA Fiber Mapping (QDFM) using phage DNA molecules. Lambda DNA molecules immobilized on APS-derivatized glass slides were hybridized with a mixture of biotin- and digoxigenin-labeled lambda DNA restriction fragments. The molecules show specific labeling after incubation with avidin-FITC and rhodamine-labeled antibodies against digoxigenin. The *insert* shows a typical molecule

found on the same slides are stretched more homogeneously, thus providing DNA fibers without need for normalization.

Expressed sequences can be mapped easily by QDFM, if each individual target extends for a few hundred base pairs or more. A common approach hybridizes small genomic DNA fragments of 1-2 kb that contain known exons onto larger genomic DNA molecules. If the cDNA sequence and some information about intron-exon boundaries are available, such small DNA fragments can rapidly be generated from genomic DNA using PCR. Figure 3C demonstrates this by mapping exon 2 of the human Band 4.1 gene onto a homologous BAC molecule. This allowed the localization of the ~2 kb exon with near kilobase precision.

Figures 3D,E illustrate the application of QDFM for measurement of BAC clone overlap. In such experiments, the DNA fiber is counterstained by a probe detected in blue. A BAC vector-specific probe (green) and a PCR-generated probe of ~1300 bp (red) that binds close to the T7 end of the vector are included to highlight the vector part of the DNA fibers (Figs. 3C,D). The two BAC clones shown in Fig. 3D overlap by approximately 80 kb and the overlapping region is close to the SP6 promoter in the BAC vector (Fig. 3D,E). These results are summarized schematically in Fig. 3E.

A rapid approach to studying the genomic organization of genes relies on direct mapping of expressed sequences. The probe DNA is isolated from cDNA clones, labeled and hybridized onto genomic DNA fibers. In the presence of blocking DNA, the cDNA probes will bind specifically to their complementary DNA targets, i.e., exons and 5- or 3-untranslated regions (UTRs) along the DNA fiber. This leaves non-coding regions (introns, 5- and 3-flanking DNA) unstained (Fig. 3F). Using FISH conditions similar to those

---

Fig. 3. Quantitative DNA Fiber Mapping (QDFM) using large insert human genomic DNA clones. A Mapping P1 clones along YAC molecules. The *arrows* indicate the distance from the respective ends. B Circular DNA molecules excised from a PFGE gel purified and stained with YOYO-1 revealed closed circular DNA molecules in the presence of linear molecules of different length. C Physical mapping of small cDNA clones in larger genomic intervals. DNA fibers (blue) prepared from a BAC clone were hybridized with a ~2 kb insert of a plasmid containing exon 2 of the human Band 4.1 gene (red). D, E Determination of overlap between linked BAC clones. A probe prepared from BAC # 103 (red) was hybridized onto DNA fibers prepared from BAC #97 (blue). E shows a schematic representation. D Mapping the genomic organization of expressed sequences. Here, BAC DNA fibers (blue) were hybridized with a ~5 kb cDNA probe (red). Three hybridization domains representing larger exons and the 3 UTR were detected on the DNA fibers. The green probe in C, D and F delineates the BAC vector

operator errors and undesirable images such as broken molecules or insufficiently stretched fibers.

References

Cassel MJ, Munné S, Fung J, Weier H-UG (1997) Carrier-specific breakpoint-spanning DNA probes for pre-implantation genetic diagnosis [PGD] in interphase cells. Hum Reprod 12:2019-2027

Cheng J-F, Weier H-UG (1997) Approaches to High Resolution Physical Mapping of the Human Genome. Biotechnology International, C. F. Fox and T.H. Connor, (Eds.), Universal Medical Press, San Francisco, pp. 149-157.

Cohen D, Chumakov I, Weissenbach J (1993) A first-generation physical map of the human genome. Nature 366:698-701

Duell T, Wang M, Wu J, Kim U-J, Weier H-UG (1997) High Resolution physical map of the immunoglobulin lambda variant gene cluster assembled by quantitative DNA fiber mapping. Genomics 45:479-486

Duell T, Nielsen LB, Jones A, Wang M, Young SG, Weier H-UG (1998) Construction of two near-kilobase resolution restriction maps of the 5' regulatory region of the human apolipoprotein B gene by Quantitative DNA Fiber Mapping (QDFM). Cytogenet Cell Genet 79:64-70

Hu J, Wang M, Weier H-UG, Frantz P, Kolbe W, Olgletree DF, Salmeron M (1996) Imaging of single extended DNA molecules on flat (aminopropyl)triethoxysilane-mica by atomic force microscopy. Langmuir 12:1697-1700

Wang M, Duell T, Gray JW, Weier H-UG (1996) High sensitivity, high resolution physical mapping by fluorescence in situ hybridization [FISH] on to individual straightened DNA molecules. Bioimaging 4:1-11

Weier H-UG, Wang M, Mullikin JC, Zhu Y, Cheng J-F, Greulich KM, Bensimon A, Gray JW (1995) Quantitative DNA fiber mapping. Hum Mol Genet 4:1903-1910

Weier H-UG, Munné S, Fung J (1999) Patient-specific probes for preimplantation genetic diagnosis (PGD) of structural and numerical aberrations in interphase cells. J Assist Reprod Genet 16:182-189

Pharmacia Biotech Inc., 800 Centennial Ave., P.O. Box 1327 Piscataway, NJ 08855-1327, USA; phone (800) 526 3593, fax (800) 329 3593, http://www.biotech.pharmacia.com

Research Genetics, Inc., 2130 Memorial Pkwy, SW, Huntsville, AL 35801, USA; phone (205) 533 4363, fax (205) 536 9016, http://www.resgen.com/

Roche Molecular Biochemicals, 9115 Hague Rd., P.O. Box 50414, Indianapolis, IN 46250-0414, USA; phone (800) 428-5433; fax (800) 428-5433

Sigma, P.O. Box 14508, St. Louis, MO 63178, USA; phone (800) 325-3010, fax (800)325-5052

Thermatron Industries, Inc., 291 Kollen Park Drive, Holland, MI49423, USA; phone (616) 393-4580, fax (616)392-5643

Vector Laboratories, Inc., 30 Ingold Road, Burlingame, CA 94010, USA; phone (415) 697-3600, fax (415)697-0339

VWR Scientific, 1310 Goshen Pkwy., West Chester, PA 19380, USA; phone (800)932-5000, fax (610) 436-1761; email solutions@vwrsp.com; http://www.vwrsp.com/

Vysis, Inc., 3100 Woodcreek Drive, Downers Grove, IL, USA: phone (800) 553-7042, fax (630) 271-7138

Abbreviations

AFM	atomic force microscopy
BAC	bacterial artificial chromosome
CEPH	Centre des Études du Polymorphisms Humain, Paris, France
LMP	low melting point
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
QDFM	quantitative DNA fiber mapping
UTR	untranslated region
YAC	yeast artificial chromosome
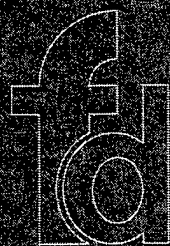
FISH in cancer diagnosis and prognostication: from cause to course of disease

*Heinz-Ulrich G Weier[†], Karin M Greulich-Bode, Yuko Ito,
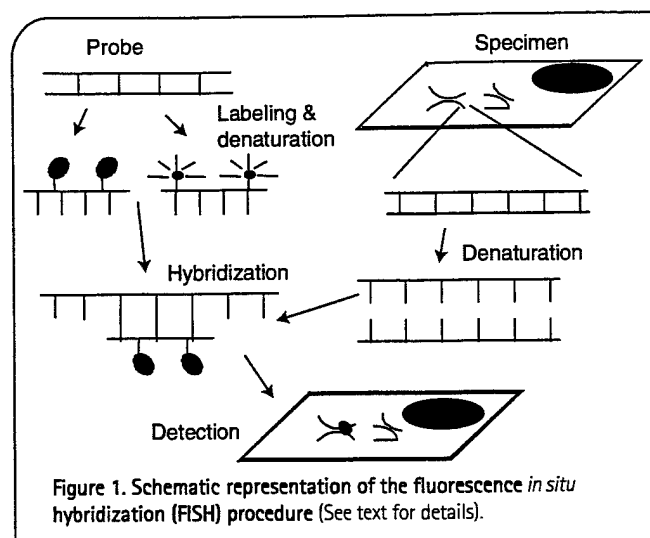Robert A Lersch and Jingly Fung*

The last 20 years have witnessed an astounding evolution of cytogenetic approaches to cancer diagnosis and prognostication. Molecular techniques and, in particular, nonisotopically-labeled nucleic acid probes and fluorescence *in situ* hybridization (FISH)-based techniques have replaced the costly and potentially dangerous radioactive techniques used in research and the clinical detection of genetic alterations in tumor cells. Fluorescent DNA probes also enabled the screening for very subtle chromosomal changes. Clinical laboratories now choose from a growing number of FISH-based cytogenetic tests to support physician's diagnoses of the causes and the course of a disease. Depending on the specimen, state-of-the-art FISH techniques allow the localization and scoring of 10–24 different targets and overcome previous problems associated with target colocalization and detection system bandwidth. FISH-based analyses have been applied very successfully to the analysis of single cells and have demonstrated the existence of cell clones of different chromosomal make-up within human tumors. This information provides disease-specific information to the attending physician and should enable the design of patient-specific protocols for disease intervention.

Most tumors are comprised of cells that escape the tightly regulated cellular life-cycle of generation, differentiation, senescence and death. Typically, genetic changes accumulate during neoplastic development and provide tumor cells with unusual growth and proliferative characteristics, most of which are inscribed in the heritable genetic code of the cells. Often referred to as mutations, these heritable changes provide genetic markers by which tumor cells might be identified and differentiated from surrounding normal cells. However, a precise determination of a normal or abnormal genetic make-up of cells often proved difficult because cells were found in different stages of the cellular growth cycle or changes were so subtle that only very few nucleic acid bases were involved.

As we learn more about the genetic changes underlying neoplastic deviation, we are also challenged to develop technologies to rapidly identify cells presenting disease-specific markers. Fluorescence *in situ* hybridization (FISH) is a nonisotopical method to visualize the location of nucleic acid sequences in cells, cell organelles, tissue sections or whole mounts of small animals. As shown schematically in FIGURE 1, FISH is based on the preparation of a nucleic acid probe labeled with a fluorescent or immunogenic hapten followed by hybridization of the probe to a DNA target rendered single-stranded by denaturation. The most simple and thus, most common way of denaturing double-stranded nucleic acids is by heating the substance above the temperature required to break the hydrogen bonds that hold together the double helix. The probe is then allowed to anneal to its complimentary nucleic acid target sequence for up to several hours and unbound probe molecules are removed by stringent washes. Most fluorochrome-labeled probes can then be seen

Figure 1. Schematic representation of the fluorescence *in situ* hybridization (FISH) procedure (See text for details).

directly in a fluorescence microscope. The detection of probes labeled with nonfluorescent haptens, such a biotin, digoxigenin or dinitrophenyl (DNP) typically involves the application of antibodies or affinity reagents, such as avidin or streptavidin, which carry fluorochromes for detection.

In the subsequent sections of this review, we will describe FISH applications on:

- Detection of specific numerical chromosome aberrations in tumor cells to aid diagnosis and tumor staging, to provide guidance in therapeutic decisions as well as to detect minimal residual disease

- Detection of specific structural chromosome aberrations associated with tumor development

- Genome-wide screening for numerical and structural chromosome aberrations to identify novel genes involved in the onset and progression of tumors

State-of-the-art FISH technology

Today's broad acceptance of FISH technology is a consequence of more than a decade of progress in two important areas. First, a vastly increased number of DNA probes and probes combinations are now available to aid researchers and clinicians in specific diagnostic investigations [1,2]. Second, innovative probe labeling techniques and significant advances in optical microscopy have helped to evolve FISH to the point where between 12–24 different DNA targets can be analyzed simultaneously on a routine basis [3–5]. The following paragraphs summarize the recent developments and highlight applications.

DNA repeat probes

Early applications of FISH involved the use of DNA probes that bind specifically to highly repeated DNA segments, such as the extensive arrays of satellite DNA found at the chromosomal centromeres and other heterochromatic regions [6]. The typical chromosome-specific target would be a large number of almost identical copies of the DNA repeat arranged in tandem [6,7]. Often, a single, small probe sequence is sufficient to label

extensive parts of a chromosome as shown for the detection of the Y chromosomes in different species using probes of about 100–200 bp [8–10]. Strong signals are generated when the small, identical probe molecules find hundreds or even thousands of binding sites in close proximity. This also allows the detection of highly iterated tandem repeats using synthetic oligonucleotides, thus circumventing time-consuming molecular cloning, DNA preparation and labeling steps. If the probe and target are distinctly different from the rest of the genome and cross-hybridization is neglectible, probes can be applied at a vast molar excess over target sites and hybridization times may be as short as a few minutes.

Locus-specific DNA probes

The term 'locus-specific DNA probe' (LSP) refers to a single probe molecule or collection of labeled nucleic acid fragments that bind to a limited, single copy region of the genome. This typically contains a gene, a translocation breakpoint or another relevant DNA sequence. Target sizes of LSPs range from about 14 kb for lambda phage-derived probes [11] to more than a million basepairs (1 Mbp) for probes prepared from large yeast artificial chromosomes (YACs) [11–13]. LSPs have gained importance in the analysis of inter- or intrachromosomal rearrangements as well as the detection of small terminal deletions [13–16]. Access to LSPs is provided through commercial sources, such as Vysis Inc., Downers Grove, IL, USA, which offers a range of probes. These include numerous probes for known oncogenes or common translocations breakpoints. While not-for-profit organizations, such as the laboratories of Drs M Simon and J Korenberg in Los Angeles prepared extensive panels of physically mapped probes and make them available to the scientific community at minimal cost [17–20], companies, such as Research Genetics of Huntsville, AL and Children's Hospital Oakland Research Institute (CHORI, Oakland CA 94609, USA) offer a fee-for-service to screen large size insert genomic DNA libraries for clones containing a gene or sequence of interest [101–110]. As demonstrated below, multicolor FISH with LSPs allows assessment of the frequency of calls carrying specific aberrations known to be associated with tumorigenesis, analysis of the series of genetic changes that occur during tumor evolution and correlation between genotype and phenotype.

Whole chromosome painting probes

Whole chromosome painting (WCP) probes are collections of labeled nucleic acid fragments that have sequence homology with regions of the genome distributed over an entire chromosome or parts thereof. These probes are typically composed of a large number of different sequences and increasing the probe complexity, i.e., the fraction of the target that is represented by probe molecules, leads to more homogeneous staining. Different strategies including chromosome enrichment by fluorescence-activated chromosome sorting (FACS), interspersed repeat sequence (IRS) or arbitrarily-primed DNA amplification and microdissection have been applied to generate WCPs [21,22]. The main application of WCPs is the detection of trans-

locations involving nonhomologous chromosomes. WCPs are now commercially available for all human and mouse chromosomes and probes labeled with different fluorochromes can be combined to delineate as many as 24 different chromosomes. This technique, termed 'multiplex-fluorescence *in situ* hybridization' (M-FISH) [23] or spectral karyotyping (SKY) [3] will be discussed in the next chapter.

Recent advances in probe labeling & detection techniques

Labeling of nucleic acid probes has never been easier. Various companies offer kits that allow even the novice to label the DNA probes used in the planned FISH experiments. The reporter molecules of choice are fluorochromes, which if bound in sufficient quantity and density, can be observed in the fluorescence microscope without further signal amplification. In the early days of FISH, most probe DNA was labeled enzymatically by either random priming or nick translation. Today, a researcher planning to prepare his or her own probes can chose between enzymatical or chemical labeling techniques to modify the DNA [24,25].

Many projects, using such techniques as M-FISH [23] or SKY [3], require the use of relatively large amounts of multiple fluor- or hapten-labeled nucleotides for the preparation of DNA probes. Such a requirement makes these experimental approaches very expensive but the cost of such nucleotides can be reduced significantly by purchasing the chemical precursors, fluor or hapten succinimidyl esters and 5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate (AA-dUTP) and performing simple coupling and purification reactions [26].

The way in which DNA probes are labeled has changed and the modalities of their detection have also undergone major evolution. Early probe detection systems involved nonfluorescent haptens and some kind of antibody sandwiching technique to couple fluorescent molecules to the hybridized DNA probes [21]. While these detection schemes are still widely used, novel signal amplification schemes, such as rolling circle amplification [27], have been developed to boost the fluorescence signals so that even faint signals can be detected with ease. Another very efficient technique involves the binding of an enzyme complex to the hybridized probe followed by precipitation of a fluorescent substrate [28,29]. Under normal conditions, either amplification system increases signal strength by a factor of 10–30, allowing the detection of very faint signals derived from small DNA targets [30].

An increasing number of fluorescent reporter molecules can be identified by virtue of their fluorescence emission spectrum leading to the development of a variety of multicolor schemes for the detection of numerical and structural chromosome aberrations. Some groups use optical filter-based microscope detection systems [21,31–34] for multicolor FISH to excite one fluorochrome at a time. Other laboratories [3,35,36] favor a system in which a multicolor filter or mirror provides the light for the simultaneous excitation of several fluorochromes and the emission filter is replaced by an optical interferometer (FIGURE 2). This spectral imaging system combines
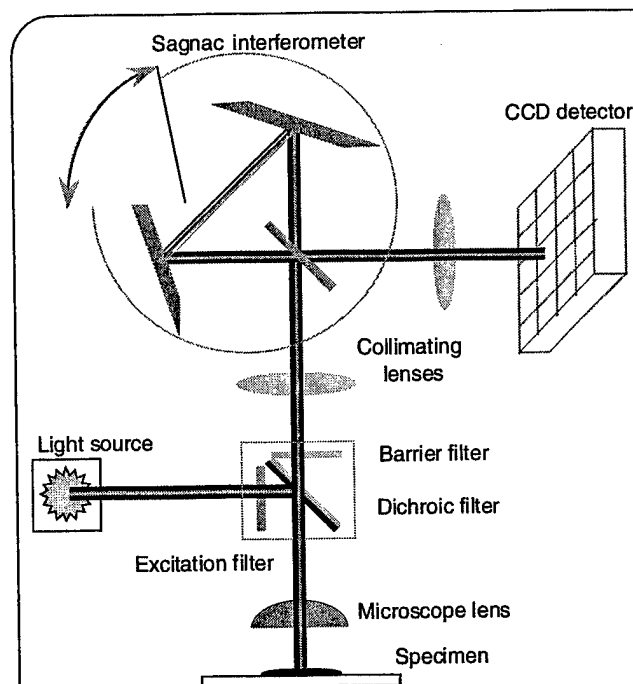

Figure 2. Schematic diagram of the optical pathway of the SpectraCube System (Image courtesy of Applied Spectral Imaging Ltd., Migdal Ha'Ermek, Israel).

the techniques of fluorescence optical microscopy, charged coupled device imaging, Fourier spectroscopy and software for digital image analysis. The power of this technology has been demonstrated by specific staining of all 24 human chromosomes in metaphase spreads, termed SKY [3,37].

The recent work of Fung *et al.* extended the application of spectral imaging to the cytogenetic analysis of interphase cell nuclei [4,35]. Five to six primary reporter molecules and a coloring scheme in which chromosome-specific probes are identified by the relative amount of each reporter molecule (termed ratio labeling) allowed scoring of up to 12 different chromosomes in a variety of interphase cell types [35]. The number of targets that can be accurately scored at the same time appears to be limited not by the labeling scheme but by the procedures available to homogeneously spread all the DNA so that spatial overlap of hybridization domains is minimized. Developed primarily for the analysis of single cells in *in vitro* fertilization and prenatal diagnosis, this technology should be easily adaptable to applications in tumor diagnosis.

Selected applications of FISH in cancer research & detection of minimal residual disease

Extra chromosomes as hallmarks of tumor cells

Trisomy 8 is observed in a significant fraction of patients with chronic myeloid leukemia (CML) [38]. In these cases, the presence of the extra chromosome 8 allows easy identification of the tumor cells and assessment of their frequency in mixed cell populations [39]. This information may become diagnostically useful and guide further therapeutic decisions. During therapy of leukemic diseases, for example, success of a specific treatment

regimen may depend on detecting early changes in the relative fraction of malignant cells in blood or bone marrow samples. FISH analysis with chromosome-specific DNA probes facilitates the differentiation between normal diploid cells and aneuploid tumor cells *via* the analysis of uncultured interphase cells. This not only reduces the time and effort required to prepare metaphase spreads for banding analysis but it also minimizes selection artifacts that might occur during cell culture [40,41]. FIGURE 3A illustrates the detection of tumor cells carrying an extra chromosome 8 among diploid bone marrow cells from a CML patient with a (47, XY, +8) tumor karyotype. Tumor cells can be identified rapidly by the presence of three hybridization signals per nucleus (FIGURE 3A, arrow).

Numerical chromosome aberrations are not limited to tumors of the hematopoietic system and they are found in a variety of solid tumors, among them renal cell cancers [42]. Metaphase spreads from these tumor cells are often difficult to obtain, thus preventing tumor studies by conventional G-banding analyses. The application of DNA repeat probes is often the method of choice for analysis of these tumors [43] and identification of tumor cells. FIGURES 3B–C show the example of interphase cells from a kidney tumor. Apparently, tumor cells are either near diploid or near tetraploid with extra copies of chromosome 8 (FIGURE 3C).

Detection of structural alterations in interphase cells

Structural chromosome alterations without gains or loss of DNA, such as reciprocal translocations, might alter the expression of oncogenes or tumor suppressor genes thus confer growth advantages to tumor cells. FISH with locus-specific probes that flank or span the breakpoint region are powerful tools to detect tumor cells as demonstrated for the bcr/abl translocation in CML [44,45]. FIGURE 3D–J illustrates the two complementary approaches. In this figure and all remaining figures, square marks next to a hybridization signal denote a red signal and triangular solid marks denote green signals. Circular open marks denote centrometic marker signals and arrows denote hybrid (red/green) signals on the same chromosome that cannot be distinguished with black and white images. Probes that span the breakpoint region can be labeled in different, i.e., chromosome-specific colors and hybridized in combination with DNA repeat probes (FIGURE 3D). As shown in (FIGURE 3F–G, cells from an individual carrying a balanced translocation t(3;4) can be analyzed for the presence and number of normal and derivative chromosomes. The probes prepared from yeast artificial chromosomes (YAC's) carry large inserts of human genomic DNA [12,13,46,47] and appear as single color hybridization domains after hybridization to normal chromosomes (FIGURE 3E and domains marked by arrowheads in FIGURE 3F). Derivative chromosomes, on the other hand, produce domains of closely spaced red and green fluorescence (arrows in FIGURE 3F,G) which can be distinguished easily from the single color domains associated with nonrearranged loci. The inclusion of one centromeric probe (marked with and open circle in this example) allows unambiguous identification
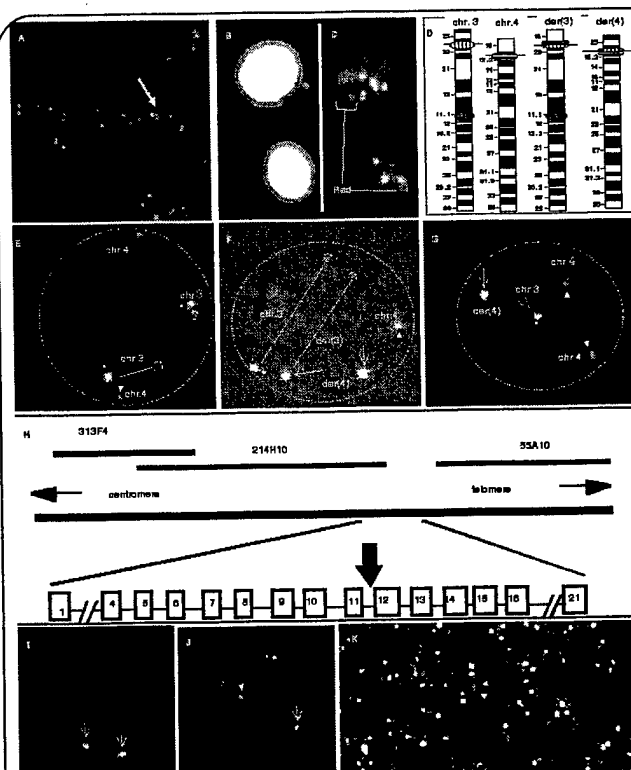
Figure 3. Locus-specific probes (LSP) detect chromosomal aberrations in interphase cells: (A) A chromosome 8-specific α satellite DNA probe identifies tumor cells in bone marrow aspirates from a leukemia patient with a tumor cell karyotype of (47,XY,+8) (arrow); (B: DAPI image, C: FISH image) In (C) combined hybridization of probes for chromosomes 8 (red; 5 spots indicated with lines) and 12 (green; all other spots) demonstrates the presence of cell clones of with extra copies of chromosomes 8 in interphase tumor cells in this case of a patient with kidney tumor; (D) A simplified hybridization scheme to detect structural and numerical chromosome alterations with probes spanning translocation breakpoints. A green probe spans a breakpoint region on chromosome 3 (oval with vertical stripes) and a probe detected in red spans the breakpoint on chromosome 4 (oval with horizontal strips). While normal chromosomes cause single color hybridization domains in interphase cells, the derivative chromosomes produce hybridization domains with adjacent red and green fluorescence (two closely adjacent ovals). Blue probes mark the centromeres of chromosome 3 thus allowing an accurate enumeration of the different chromosome types (stippled ovals); (E) Hybridization of locus-specific probes for chromosomes 3 and 4 to normal interphase cells produces large, single color domains representing the respective targets on chromosome 3 (green, square) and 4 (red, triangle), respectively; (F) Hybridization of the probes shown in (D) to cells derived from an individual carrying a reciprocal translocation t(3;4) shows single-color 'normal' domains (red, triangle; green, square) and the presence of translocation chromosomes in form of associated red–green domains (arrows); (G) Numerical as well as structural chromosome aberrations can be detected with locus-specific probes. Here, hybridization of the chromosome 3/4 probe set to embryonic cells demonstrates one copy of chromosome 3 (square), two copies of chromosome 4 (triangles) and one copy of a der(3) chromosome (arrow), i.e., aneusomy with respect to the distal parts of chromosomes 3p and 4p; (H) Hybridization scheme to detect ret gene rearrangements in interphase cells. Green-labeled probes bind to the 5'-end of the ret gene (313F4, 214H10), while a red labeled probe in binds to the 3'-end of the gene (55A10). The common breakpoint maps into intron 11 of the ret gene. (I) Hybridization of the ret-specific probes to interphase cell nuclei from normal donors reveals two-hybridization domains with associated red–green staining (arrows); (J) Hybridization of the ret-specific probes to interphase cell nuclei from the thyroid cancer cell line TPC-1 copy shows one red–green domain representing a nonrearranged of the ret gene (arrow) and separated green and red domains (red, triangle; green, square) indicating the rearranged copy of the second homologue; (K) Amplification of the c-myc gene in a case of melanoma. A touching imprint preparation was hybridized with a probe specific for c-myc (red, a center cell marked with squares at green signals), which maps to 8q24 and a chromosome 8-specific centromeric DNA repeat probe as control (Vysis CEP 8, green, a center cell marked with squares at green signals). Generally, the green signals are bright and fat; the red signals are smaller and less intense. (Image courtesy of J. Utikal, Department of Dermatology, University of Ulm, Ulm, Germany). (See online version of this article for color figures).

of the derivative chromosomes [48]. Besides the identification of structural chromosome aberrations, hybridization of

breakpoint spanning probes also allows the detection of numerical aberrations or aneuploidies [12,13]. In the example presented in FIGURE 3G, the reciprocal translocation in the father's germline interfered with the proper segregation of chromosomes in meiosis and led to aneuploid offspring [13,48]. In this case, hybridization involving breakpoint spanning probes as well as a chromosome enumerator (centromeric) probes helped to detect partial aneusomy in the embryonic cells [48]. The very same scheme can be applied to characterize cells from carriers of the translocation t(11;22), which is the most common translocation in humans and indicates an increased tumor risk in carriers [49].

The detection of structural rearrangements in tumor interphase cells with probes that flank one or both translocation breakpoints was first described for the above mentioned bcr/abl translocation involving genes on human chromosomes 9 and 22 [2,45]. FIGURE 3H shows a similar scheme developed to detect translocations that activate the ret proto-oncogene in papillary thyroid cancer (PTC) [50]. The common breakpoint in ret-activating translocations in PTC maps to intron 11 just upstream of the catalytic domain of this tyrosine kinase. As outlined in FIGURE 3H, we prepared probes that bind to either the 5'-end (clones 313F4 and 214H10) or the 3'-end of the ret gene (clone 55A10) and detected the bound probes with green or red fluorochromes, respectively. Mononuclear white blood cells from normal donors display the expected red/green domains (FIGURE 3I, arrows), whereas cells from the PTC cell line TPC-1 show one red/green 'normal' domain (arrow) and one set of separated red and green hybridization domains (FIGURE 3J). This was expected since TPC-1 is known to carry a rearranged copy of the ret gene [50].

Application of FISH in tumor prognostication

Numerous studies have shown that tumor development is accompanied by at least two changes:

• A change in the way cells interact with their environment *via* membrane-bound receptors

• A change in how signals originating from these receptors are transduced from the cell membrane to the cytoplam and the cell nucleus where it will alter the levels of expression of particular genes

Among the hundreds of genes involved in receptor-mediated signal transduction, only a few have been shown to be aberrantly expressed in tumors. For example, overexpression of tyrosine kinase genes, due to gene amplification or changes in the regulation of gene expression, may lead to oncogenic transformation. This has been clearly documented for the erbB-2 protein, the product of the Her-2/neu proto-oncogene and other members of the erbB family, especially in breast cancer patients. In addition, many tumors have acquired structurally altered tk proteins as well as an abnormal expression pattern through *de novo* mutational events. In cases where chromosomes have become rearranged, the catalytic domain of a tk gene can be fused to the amino terminal of another protein, thus creating a new, transforming activity as well as a new expression

pattern. The above-mentioned activating ret rearrangement is just one example. Other well known examples of this mechanism of oncogene activation are the previously discussed bcr/abl-fusion protein in CML with t(9;22) and the activation of the receptor tyrosine kinase trk in papillary thyroid cancer.

Overexpression of particular genes, such as the insulin-like growth factor receptors (IGF-IRs), the epidermal growth factor receptor (EGFR or erbB) family of receptors, focal adhesion kinase (FAK) or the proto-oncogenes ret and Nyk/mer have been shown to correlate with progression to a more malignant phenotype in a variety of tumors. Other genes that have often been found amplified in solid tumors appear to be related to increased gene expression, such as the c-myc gene which maps to the long arm of human chromosome 8. Interphase FISH with locus-specific probes is able to accurately determine the number of copies of a gene per cell. Such detailed knowledge about gene amplification coupled with additional measurement of gene expression might increase our understanding of how tumors grow. Such knowledge potentially leads to the design of assays, which will allow us to perform more accurate staging of tumors and predict the course of tumor development, i.e., its capacity to grow, invade and spread to other sites.

As an example, FIGURE 3K illustrates the detection of gene amplifications in skin cancers. Tumor cells were transferred from a melanoma sample onto glass microscope slides by the touching imprint method and hybridized with a probe specific for the c-myc proto-oncogene (signals in FIGURE 3K a center cell marked with triangles at signals). A commercial probe that stains repeated DNA in the centromeric region of chromosome 8 (CEP 8, Vysis, Inc., labeled with green fluorochromes, a center cell marked with squares at green signals) was included to determine the number of whole chromosomes 8. Hybridization results (FIGURE 3K) confirm high level amplification of the c-myc gene without extra copies of chromosome 8 in these cells. This patient-specific information combined with results of population-based retrospective studies correlating c-myc gene amplification with tumor metastasis and average time of disease-free survival might allow the attending dermatologist/oncologist to prepare a more individualized therapy regimen [51].

WCP in the analysis of metaphase spreads

Whole chromosome painting (WCP) is a rapid technique to detect translocations involving nonhomologous chromosomes. High quality painting probes delineate the target chromosome from one end to the other, while the hybridization of nonspecific repeats, such as interspersed repeats or centromeric clusters of satellite DNA is blocked by addition of an excess of unlabeled repeat DNA to the hybridization mixture [22].

Simple chromosome painting experiments use only one or two WCP probes as shown in FIGURE 4A for the delineation of chromosomes 1 (square) or 8 (triangle). This is often cost-efficient and confirms a suspected rearrangement, such as the translocation t(1;9)(p36;q13) in a case of follicular lymphoma with an
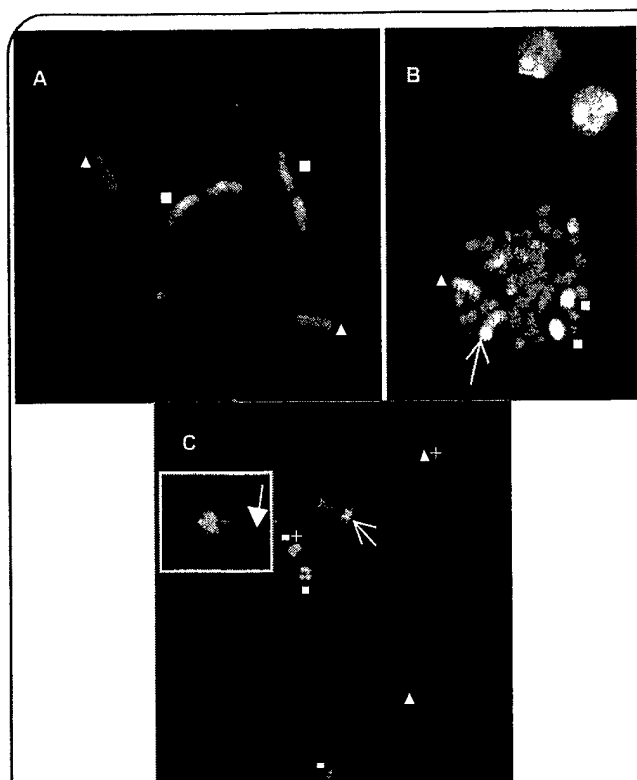
Figure 4. Whole chromosome painting (WCP) probes in the analysis of metaphase cells: (A) Dual-color hybridization delineating normal chromosomes 1 (square) and 8 (triangle) in a normal human metaphase spread; (B) Dual-color hybridization of WCP probes for chromosome 1 (square) and chromosome 9 (triangle) delineates normal and translocation chromosomes (arrow) in a leukemia case carrying a t(8;14) and a t(1;9). (Image courtesy of Dr. I Wlodarska, Leuven, Belgium); (C) Structural alterations in a thyroid cancer cell line lead to rearrangements of chromosomes 3 (square) and 17 (triangle). A dual-color hybridization reveals normal homologues (a plus mark next to the square or triangle), derivatives of either chromosomes 3 or 17 (square or triangle respectively) and a marker chromosome (solid arrowhead within insert).

additional t(14;18) shown in FIGURE 4B (the arrows point at rearranged chromosome). Using two WCP probes labeled with different reporter molecules also allows detection of translocations involving one or several stained chromosomes and any of the unstained chromosomes. FIGURE 4C illustrates this with an example from the analysis of a follicular thyroid cancer cell line. A chromosome 3-specifc WCP probe (triangle) was combined with a WCP probe for human chromosome 17 (square). An intact chromosome 3 and an intact chromosome 17 were detected. In addition, the metaphase spreads showed multiple rearrangements (open arrowhead) involving either chromosome 3 or chromosome 17 and unstained and as yet unidentified chromosomes. A marker chromosome (FIGURE 4C, insert) was found comprised of chromosome 3 and chromosome 17 material in addition to a small amount of DNA from an as yet unidentified chromosome. The unidentified material at one end of this marker chromosome was visualized by DAPI which counterstains all DNA (FIGURE 4C, solid arrowhead within insert). Chromosome painting is the method of choice to define larger breakpoint intervals in translocations like the ones shown here. Further detailed mapping of the

breakpoints can then be performed by 'chromosome walking' using LSPs [12,13,46–48] or sets of probes spread along the target chromosomes in narrow distances [32,52,53].

SKY & M-FISH analyses

Complex rearrangements in tumor cells are mapped rapidly by using tumor metaphase cell preparations and combining a larger number of WCP probes. If the unambiguous classification of all chromosomes in a metaphase spread is required, the user can choose between SKY or filter-based M-FISH. The SKY approach is able to resolve fluorescence spectra with a resolution of about 10 nm, thus it records the equivalent of 30–40 distinctly different spectral images. This takes more time than recording 5–7 images with an M–FISH system and the user may want to weigh the advantages of high resolution against those of a higher throughput analysis.

FIGURE 5 illustrates the SKY analysis of a metaphase spread prepared from the human prostate cancer cell line TSUPR1. Images were acquired with an SD200 SpectraCube^{TM} Spectral Imaging system (ASI, Inc., Carlsbad, CA). The spectral imaging system attached to a Nikon E600 microscope consisted of an optical head (Sagnac interferometer) coupled to a multi-line CCD camera (Hamamatsu). The image data were stored in a Pentium 586/300 MHz computer and analyzed by proprietary software. The multiple band pass filter set used for fluorochrome excitation was custom-designed (SKY-1, ChromaTechnology, Brattleboro, VT) to provide broad emission bands (giving a fractional spectral reading from ~ 450 nm to ~ 850 nm). Using a Xenon light source, the spectral image was generated by acquiring 80–130 interferometric frames per object. The time needed to acquire the image was less than 3 min.

The DAPI image (FIGURE 5A) helped to identify chromosomes and chromosomal breakpoint regions. Following chromosome classification based on the full 450–850nm fluorescence spectrum, individual chromosomes were assigned 'classification colors' which linked the fluorescence spectra to chromosome-specific WCP probe mixtures (FIGURE 5A) [3,37]. SKY analysis of TSUPR1 metaphases revealed a number of previously unknown translocations (t(2;8),t(3;19),t(6;7), t(6;15),t(15;18)) in the presence of several marker chromosomes (FIGURE 5D). FIGURE 5A shows the inverted image of the chromosome spread acquired through the DAPI filter and FIGURE 5B shows the WCP probe fluorescence along the same chromosomes as an RGB pseudo-color image (presented in grayscale). Based on the measurement of the complete spectrum for each point in this metaphase image, a spectral classification algorithm allowed the assignment of a defined pseudo-color to all points in the image that displayed the same spectrum. A karyotype table was obtained (FIGURE 5C) showing 84 chromosomes with six of them classified as marker chromosomes. The karyotype table (FIGURE 5D) shows the chromosomes in classification colors to the left and normal ideograms to the right. Clearly, translocations in this cell prostate cancer cell line involved the chromosomes 2, 3, 4, 5, 7, 8, 11, 15, 18 and 19.
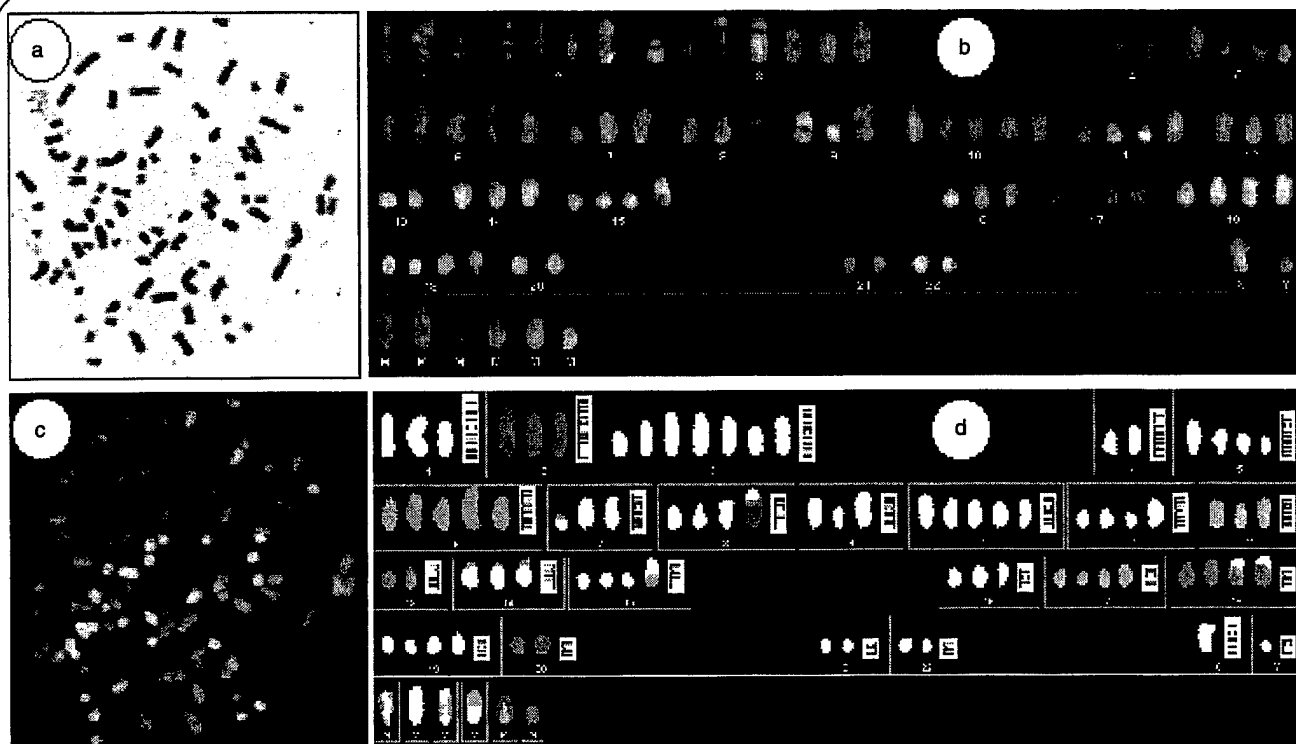
Figure 5. Spectral karyotyping (SKY) analysis of a tumor cell metaphase spread. SKY analyses of metaphase spreads from the human prostate cancer cell line TSUPR1: (a) Inverted DAPI image; (b) RGB pseudo-color image rendered in grayscale; (c) Karyotype table in grayscale; (d) Spectral karyotype showing chromosomes after assignment of classification colors to the left and normal idiograms to the right rendered in grayscale. Figures reproduced with kind permission of Springer Verlag [35].

The application of M-FISH is illustrated by the example shown in FIGURE 6. Metaphase spreads from a primary culture of cells from a female acral lentiginous melanoma (ALM) patient were hybridized with SpectraVysion probe mixture (Vysis) and analyzed using an M-FISH microscope system. The M-FISH images reveal several numerical and structural abnormalities summarized as karyotype of 44,X,+7,-10,-X,del(2q),t(1;8),t(3;22),t(6;15), t(3;22), del (2q).

Both techniques, SKY as well as M-FISH, have detection limits in the megabasepair range [54]. The most common problem with the detection of small chromosomal fragments in complex translocations is the spatial overlap of combinatorially labeled probes. As Lee et al. [54] pointed out, the juxtaposition of material from nonhomologous chromosomes frequently results in overlapping fluorescence at the interface of the translocated segments, a phenomenon also referred to as 'flaring' [55]. In most cases, the negative impact of flaring and chromosome misclassification can be addressed by subsequent hybridization using chromosome-specific WCP probes [54].

Conclusion & expert opinion

Intensive biomedical research in the last two decades has increased our understanding of genetic changes underlying tumorigenesis and progression to a more malignant tumor phenotype. The International Human Genome Project and its many associated efforts have generated resources and tools that now enable researchers to identify genetic changes at the level of single cells.

Playing a pivotal role in the genetic analysis of single cells, FISH has frequently helped to unravel the complex changes accompanying tumor invasion and metastasis. FISH owes its wide acceptance to the fact that it has become a simple procedure bringing together two main components: cells and tissue specimens (provided by the researcher or clinician) and nonisotopically-labeled probes, many of which are now commercially available. The near future is likely to see a significant increase in the number of probes cleared by the US FDA for *in vitro* diagnostic use as well as ready-to-use reagents pushing the limits of detection to smaller targets and, at the same time, increase the number of targets that can be studied simultaneously. Instrument prices are unlikely to change but increased automation and knowledge-based information systems will facilitate the molecular cytogenetic analyses and reduce costs.

The FISH-based assays will be rapid, inexpensive and require only a small number of cells, thus providing an affordable diagnostic service to the large community of cancer patients in the USA and elsewhere. Information gained by these measurements can be utilized to enhance prognostication, therapeutic decisions and patient management or to measure the effects of drugs in laboratory as well as clinical studies. We steadily increase our knowledge about the relationship between genetic alterations and the course of the disease. Determining genetic changes beside the status of biochemical and histopathological markers will enable clinicians to provide patients with a more individualized therapy.
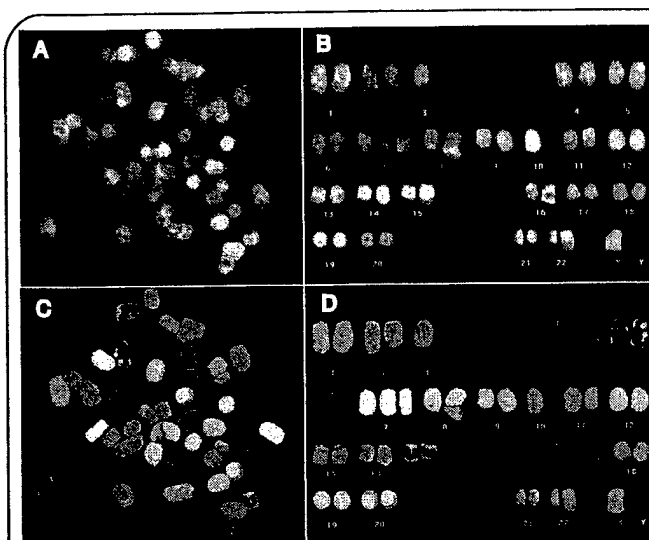
Figure 6. M–FISH analysis of human metaphase spreads reveals structural alterations in a case of acral lentiginous melanoma (ALM). Metaphase spreads from a primary culture of cells from a female ALM patient were hybridized with SpectraVysion probe mixture (Vysis, Inc.) and analyzed using an M–FISH microscope system. The M–FISH images reveal several abnormalities summarized as karyotype of 44,X,+7,-10,-X,del(2q),t(1;8),t(3;22),t(6;15), t(3;22), del (2q). **(A)** RGB colored image of the metaphase spread rendered in grayscale; **(B)** the karyotype table in in grayscale; **(C)** the metaphase image after assignment of classification colors rendered in grayscale; **(D)** the karyotype table in classification colors rendered in grayscale

Five-year view

Rapid progress of the Human Genome Project and the completion of a draft sequence of the human genome have provided a course for even more comprehensive genetic analyses in the future. As key events and changes during the progression of normal cells to malignant tumors are deciphered, FISH probes and hybridization protocols will be developed to visualize these changes in individual cells. To the clinician, this will provide important information complementing histopathological analyses and thus may guide individualized therapeutic decisions.

While multicolor FISH techniques are poised to help decipher the complex changes underlying cancer, the early detection of tumor cells or minimal residual disease as well as identification of individuals at elevated risk faces several challenges. First, FISH technology must go beyond a simple detection of the somewhat static cytogenetic changes and address the relative levels of expression of genes involved in the tumor phenotype. A typical example is the overexpression of the erbB-2 protein, the product of the Her-2/neu proto-oncogene, in various forms of cancer. There is growing evidence that gene amplification in addition to overexpression is an important prognostic factor in breast cancer and future FISH techniques should be able to render information about the levels of both, amplification as well as gene expression.

Other concerns are directed towards the amount of material needed to perform a comprehensive genetic analysis of tumor cells by FISH. The successful analysis of single blastomeres biopsied from preimplantation human embryos [47,48,56] has opened up new avenues for the analysis of small samples. Future technology developments will be geared towards the generation of a large amount of cytogenetic information from a small number of cells.

As the main thrust of research in the postgenome era shifts to proteomics, FISH techniques will need to change and accommodate immunocytochemical techniques, or minimally, be able to combine nucleic acid analyses with protein quantitation and phenotypic characterization using tumor marker-specific antibodies. Second, to conduct population studies, FISH technology needs to become standardized and automated. Present hands-on procedures must be modified to allow the processing of hundreds or even thousands of specimens within reasonable timeframes. This also calls for knowledge-based, automated analysis systems able to gather and categorize information at very high speed. While most of the necessary technology already exists, it will take continued funding and engineering ingenuity to bring the pieces together. Finally, the power of FISH-based techniques for patient diagnosis is now beginning to be harnessed. Many health professionals outside the field of molecular genetics must be trained to correctly interpret FISH data. Computer professionals, who will design and implement future software and primary care professionals, who work directly with the patients, must be trained to understand FISH data. This training may turn out to be as challenging to achieve as the technical horizons we have mapped out above.

Key issues

- FISH is non-isotopic method to localize nucleic acids, such as DNA and RNA.

- Increased application of FISH assays in the clinical practice will require thorough training of healthcare professionals.

- Probes used for FISH are labeled with nonradioactive haptens, such as biotin, digoxigenin or fluorochromes. They are stable for several years and can be disposed of without hazards. Several FISH probes labeled with different reporter molecules can be combined for multilocus analyses. Many FISH probes are now commercially available.

- FISH assays can be set up to investigate specific alterations which are typical for a particular tumor or they can be designed to screen the entire genome for alterations.

- FISH is sensitive and rapid.

- FISH results support the molecular staging of tumors, thus facilitate prognostication and individualized treatment regimens.

- FISH assays detect numerical as well as structural alterations in a wide variety of cells and tissues and, as our understanding of the cytogenetic changes underlying tumorigenesis, invasion and metastasis increases, will evolve to specifically address the etiology of each disease.

- To be able to handle a significant larger number of clinical samples, FISH techniques need to be automated. There is also a growing need to design digital imaging systems and expert computer systems for FISH analyses in the clinical laboratory.

References

Papers of special note have been highlighted as:
- of interest
- •• of considerable interest

1. Trask BJ. Fluorescence *in situ* hybridization: applications in cytogenetics and gene mapping. *Trends Genet.* 7(5), 149–154 (1991).

2. Tkachuk D, Pinkel D, Kuo W-L, Weier H-U, Gray JW. Clinical applications of fluorescence *in situ* hybridization. *Genet. Anal. Tech. Appl.* 8, 676–674 (1991).

3. Schroeck E, du Manoir S, Veldman T *et al.* Multicolor spectral karyotyping of human chromosomes. *Science* 273, 494–497 (1996).
   - • This is the first publication of SKY for the cytogenetic analysis of tumor cells.

4. Fung J, Weier HU, Goldberg JD, Pedersen RA. Multilocus genetic analysis of single interphase cells by spectral imaging. *Hum. Genet.* 107(6), 615–622 (2000).

5. Nietzel A, Rocchi M, Starke H *et al.* A new multicolor-FISH approach for the characterization of marker chromosomes: centromere-specific multicolor-FISH (cenm-FISH) *Hum. Genet.* 108(3), 199–204 (2001).

6. Willard HF, Waye JS. Hierarchical order in chromosome-specific alpha satellite DNA. *Trends Genet.* 3, 192–198 (1987).

7. Moyzis RK, Albright KL, Bartholdi MF *et al.* Human chromosome-specific repetitive DNA sequences: novel markers for genetic analysis. *Chromosoma* 95, 375–386 (1987).

8. Weier H-U, Segraves R, Pinkel D, Gray JW. Synthesis of Y-chromosome specific, labeled DNA probes by *in vitro* DNA amplification. *J. Histochem. Cytochem.* 38, 421–427 (1990).

9. Nilsson SK, Dooner MS, Weier H-U *et al.* Cells capable of bone production engraft from whole bone marrow transplants in nonablated mice. *J. Exp. Med.* 189, 729–734 (1999).

10. Yao R, Sugino IK, Greulich KM, Ishida M, Weier H-UG, Zarbin MA. Optimization of non-isotopic *in situ* hybridization: detection of Y-chromosome in paraformaldehyde-fixed, wax-embedded cat retina. *Exp. Eye Res.* 66, 223–230 (1998).

11. Weier H-UG, George CX, Greulich KM, Samuel CE. The interferon-inducible, double-stranded RNA-specific adenosine deaminase gene (DSRAD) maps to human chromosome 1q21.1-21.2. *Genomics* 30, 372–375 (1995).

12. Cassel MJ, Munné S, Fung J, Weier H-UG. Carrier-specific breakpoint-spanning DNA probes for pre-implantation genetic diagnosis [PGD] in interphase cells. *Hum. Reprod.* 12, 101–109 (1997).

13. Fung J, Munne S, Weier HU. Detection of chromosome translocation products in single interphase cell nuclei. *Meth. Cell Biol.* 64(B), 97–114 (2001).
   - •• A laboratory manual providing detailed experimental protocols.

14. Ning Y, Roschke A, Smith ACM *et al.* A complete set of human telomeric probes and their clinical applications. *Nature Genet.* 14, 86–89 (1996).

15. Knight SJ, Lese CM, Precht KS *et al.* An optimized set of human telomere clones for studying telomere integrity and architecture. *Am. J. Hum. Genet.* 67, 320–332 (2000).

16. Henegariu O, Artan S, Greally JM *et al.* Cryptic translocation identification in human and mouse using several telomeric multiplex FISH (tm-FISH) strategies. *Lab. Invest.* 81, 483–491 (2001).

17. Shizuya H, Birren B, Kim UJ *et al.* Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia Coli* using an f-factor-based vector. *Proc. Natl Acad. Sci. USA* 89, 8794–8797 (1992).

18. Kim U-J, Birren B, Sheng Y-L *et al.* Construction and characterization of a human bacterial artificial chromosome library. *Genomics* 34, 213–218 (1995).

19. Korenberg JR, Chen XN, Sun Z *et al.* Human genome anatomy: BACs integrating the genetic and cytogenetic maps for bridging genome and biomedicine. *Genome Res.* 9, 994–1001 (1999).

20. McPherson JD, Marra M, Hillier L *et al.* A physical map of the human genome. *Nature* 409, 934–941 (2001).

21. Pinkel D, Landegent J, Collins C *et al.* Fluorescence *in situ* hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocations of chromosome 4. *Proc. Natl Acad. Sci. USA* 83, 2934–2938 (1988).
   - • A landmark paper introducing the whole chromosome painting technique.

22 Weier H-U, Pinkel D, Gray J. Whole chromosome complementary probe fluorescence staining. In: *Encyclopedia of Molecular Biology and Molecular Medicine (Volume 6)*. Meyers RA (Ed.), VCH Publishers, Weinheim, Germany, 253–257 (1997).

23 Speicher MR, Ballard SG, Ward DC. Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nature Genet.* 12, 368–375 (1996).

•   This paper describes the M-FISH technology.

24 Fung J, Moronne MM, Weier H-UG. A simple blocking system for use with maleimide-labeled nucleic acid probes. *BioTechniques* 25, 390–394 (1999).

25 Gijlswijk RPM van, Talman EG, Janssen PTA *et al.* Universal Linkage System: versatile nucleic acid labeling technique. *Expert Rev. Mol. Diagn.* 1(1), 81–91 (2001).

•   This review describes Kreatech's proprietary ULS labeling techniques and provides an overview of nucleic acid hybridization and detection techniques.

26 Nimmakayalu M, Henegariu O, Ward DC, Bray-Ward P. Simple method for preparation of fluor/hapten-labeled dUTP. *BioTechniques* 28, 518–522 (2000).

27 Zhong XB, Lizardi PM, Huang XH, Bray-Ward PL, Ward DC. Visualization of oligonucleotide probes and point mutations in interphase nuclei and DNA fibers using rolling circle DNA amplification. *Proc. Natl Acad. Sci. USA* 98(7), 3940–3945 (2001).

28 Raap AK, van de Corput MP, Vervenne RA, van Gijlswijk RP, Tanke HJ, Wiegant J. Ultra-sensitive FISH using peroxidase-mediated deposition of biotin- or fluorochrome tyramides. *Hum. Mol Genet.* 4(4), 529–534 (1995).

29 Gijlswijk RP van, Zijlmans HJ, Wiegant J *et al.* Fluorochrome-labeled tyramides: use in immunocytochemistry and fluorescence *in situ* hybridization. *J. Histochem. Cytochem.* 45(3), 375–382 (1997).

30 Weier H-UG. DNA fiber mapping techniques for the assembly of high-resolution physical maps. *J. Histochem. Cytochem.* 49(8), 939–945 (2001).

31 Jossart GH, O'Brien B, Cheng J-F *et al.* A novel multicolor hybridization scheme applied to localization of a transcribed sequence (D10S170/H4) and deletion mapping in the thyroid cancer cell line TPC-1. *Cytogenet. Cell Genet.* 75(4), 254–257 (1996).

32 Chudoba I, Plesch A, Lorch T, Lemke J, Claussen U, Senger G. High resolution multicolor-banding: A new technique for refined FISH analysis of human chromosomes. *Cytogenet. Cell Genet.* 84, 156–160 (1999).

33 Greulich KM, Kreja L, Heinze B *et al.* Rapid detection of radiation-induced chromosomal aberrations in lymphocytes and hematopoietic progenitor cells by M-FISH. *Mut. Res.* 452, 73–81 (2000).

34 Nietzel A, Rocchi M, Starke H *et al.* A new multicolor-fish approach for the characterization of marker chromosomes: centromere-specific multicolor-FISH (cenm-FISH). *Hum. Genet.* 108, 199–204 (2001).

35 Fung J, Weier H-UG, Pedersen RA, Zitzelsberger HF. Spectral imaging analysis of metaphase and interphase cells. In: *FISH Technology.* Rautenstrauss B, Liehr T (Eds.), Springer Verlag, Heidelberg, Germany, 363–387 (2002).

36 Zitzelsberger H, Bruch J, Smida J *et al.* Clonal chromosomal aberrations in simian virus 40-transfected human thyroid cells and in derived tumors developed after *in vitro* irradiation. *Int. J. Cancer* 96(3), 166–177 (2001).

37 Garini Y, Macville M, du Manoir S *et al.* Spectral karyotyping. *Bioimaging* 4, 65–72 (1996).

38 Haas OA, Schwarzmeier JD, Nacheva E, Fisher P, Paietta E. Investigations on karyotype evolutions in patients with chronic myeloid leukemia. *Blut* 48, 33–43 (1984).

39 Weier H-U, Kleine H-D, Gray JW. Labeling of the centromeric region on human chromosome 8 by *in situ* hybridization. *Hum. Genet.* 87, 489–494 (1991).

40 Cremer T, Landegent J, Bruckner A *et al.* Detection of chromosome aberrations in the human interphase nucleus by visualization of specific target DNAs with radioactive and non-radioactive *in situ* hybridization techniques: diagnosis of trisomy 18 with probe L1.84. *Hum. Genet.* 74, 346–352 (1986).

41 Pinkel D, Gray JW, Trask B, Engh G van den, Fuscoe J, Dekken H van. Cytogenetic analysis by *in situ* hybridization with fluorescently labeled nucleic acid probes. *Cold Spring Harb. Symp. Quant. Biol.* 51, 151–157 (1986).

42 Zitzelsberger HF, Szücs S, Weier HU *et al.* Numerical abnormalities of chromosome 7 and polyploidization in human prostate cancer detected by fluorescence *in situ* hybridization (FISH) on paraffin-embedded tissue sections with centromere-specific DNA probes. *J. Path.* 172, 325–335 (1994).

43 Weier HU, Rosette CD, Matsuta M, Zitzelsberger H, Matsuta M, Gray J. Generation of highly specific DNA hybridization probes for chromosome enumeration in human interphase cell nuclei: isolation and enzymatic synthesis of α satellite DNA probes for chromosome 10 by primer directed DNA amplification. *Meth. Mol. Cell Biol.* 4, 231–248 (1994).

44 Arnoldus EP, Wiegant J, Noordermeer IA *et al.* Detection of the Philadelphia chromosome in interphase nuclei. *Cytogenet. Cell Genet.* 54, 108–111 (1990).

45 Tkachuk DC, Westbrook CA, Andreeff M *et al.* Detection of bcr-abl fusion in chronic myelogeneous leukemia by *in situ* hybridization. *Science* 250, 559–562 (1990).

46 Fung J, Munné S, Duell T, Weier H-UG. Rapid cloning of translocation breakpoints: from blood to YAC in 50 days. *J. Biochem. Mol. Biol. Biophys.* 1, 181–192 (1998).

47 Lersch RA, Fung J, Munné S, Pedersen RA, Weier H-UG. Case-specific, breakpoint-spanning DNA probes for analysis of single interphase cells. *Genet. Test.* 4, 273–278 (2000).

•   This paper summarizes the detection of structural and numerical chromosome aberrations in single blastomeres.

48 Munné S, Fung J, Cassel MJ, Márquez C, Weier H-UG. Preimplantation genetic analysis of translocations: case-specific probes for interphase cell analysis. *Hum. Genet.* 102, 663–674 (1998).

49 Fung J, Munné S, Garcia J, Kim U-J, Weier H-UG. Reciprocal translocations and infertility: molecular cloning of breakpoints in a case of constitutional translocation t(11;22)(q23;q11) and preparation of probes for preimplantation genetic diagnosis (PGD). *Reprod. Fertil. Devel.* 11, 17–23 (1999).

50 Jossart GH, Greulich KM, Siperstein AE, Duh Q, Clark OH, Weier H-UG. Molecular and cytogenetic characterization of a t(1;10;21) translocation in the human papillary thyroid cancer cell line TPC-1 expressing

the ret/H4 chimeric transcript. *Surgery* 118(6), 1018–1023 (1995).

51   Kraehn GM, Utikal J, Udart M *et al.* Extra c-myc oncogene copies in high risk cutaneous malignant melanoma and melanoma metastases. *Br. J. Cancer* 84(1), 72–79 (2001).

52   Weier HUG, O'Brien B, Duell TH *et al.* High-resolution, multi-locus karyotype analysis with 'Chromosomal Rainbows (CR)': a novel technique applied to rapid characterization of rearranged chromosomes in primary cultures and cell lines from thyroid cancers. *Am. J. Hum. Genet.* 59(Suppl.), A50 (1996).

53   Lemke J, Chudoba I, Senger G *et al.* Improved definition of chromosomal breakpoints using high-resolution multicolor banding. *Hum. Genet.* 108(6), 478–483 (2001).

54   Lee C, Gisselsson D, Jin C *et al.* Limitations of chromosome classification by multicolor karyotyping. *Am. J. Hum. Genet.* 68(4), 1043–1047 (2001).

55   Lu YJ, Morris JS, Edwards PA, Shipley J. Evaluation of 24-color multifluor-fluorescence *in situ* hybridization (M-FISH) karyotyping by comparison with reverse chromosome painting of the human breast cancer cell line T-47D. *Chrom. Res.* 8(2), 127–132 (2000).

56   Wells D, Sherlock JK, Handyside AH, Delhanty JDA. Detailed chromosomal and molecular genetic analysis of single cells by whole genome amplification and comparative genomic hybridization. *Nucl. Acids Res.* 27(4), 1214–1218 (1999).

Websites

101   www.ornl.gov/hgmis/

102   www.csmc.edu/genetics/korenberg/korenberg.html

103   www.chori.org/bacpac

104   www-genome.wi.mit.edu/

105   www.resgen.com/resources/index.php3

106   www.sanger.ac.uk/Teams/Team63/CloneRequest

107   www.vysis.com/

108   www.cytocell.com/home.html

109   www.molecularprobes.com/

110   http://probentis.de

Affiliations

- *Heinz-Ulrich G. Weier, Ph.D.*
  ugweier@lbl.gov
- *Yuko Ito, Ph.D.*
  Yito@lbl.gov,
- *Robert A. Lersch, Ph.D.*
  RALersch@lbl.gov
  Department of Subcellular Structure,
  Life Sciences Division, MS 74-157,
  University of California,
  E.O. Lawrence Berkeley National Laboratory,
  1 Cyclotron Road, Berkeley,
  CA 94720, USA
  Tel.: + 1 510 486 5363
  Fax: +1 510 486 5343
- *Karin M. Greulich-Bode, Ph.D.*
  Department Genetics of Skin Carcinogenesis,
  German Cancer Research Center, Dept. B0900
  Im Neuenheimer Feld 280,
  D-69120 Heidelberg, FRG
  Tel.: + 49 6221 42 3433
  Fax: +49 6221 42 3457
  kgreulich@debitel.net
- *Jingly Fung, Ph.D.*
  Reproductive Genetics Unit,
  Department of Obstetrics, Gynecology, and
  Reproductive Sciences,
  University of California,
  533 Parnassus, San Francisco,
  CA 94143-0720, USA
  Tel.: + 1 415 476 8517
  Fax: +1 415 476 6145
  jlfung@itsa.ucsf.edu